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# Development of covalent NLRP3 inflammasome inhibitors: chemistry and biological activity

Massimo Bertinaria,\* Simone Gastaldi, Elisabetta Marini, Marta Giorgis

*Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria  
9 – 10125 Torino, Italy*

**Keywords:** NLRP3 inflammasome; NLRP3 inhibitors; covalent drugs; irreversible inhibitors, drug design.

## Abstract

The NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome is the best recognized and most widely implicated regulator of caspase-1 activation. It is a key regulator of innate immune response and is involved in many pathophysiological processes. Recent evidences for its inappropriate activation in autoinflammatory, autoimmune, as well as in neurodegenerative diseases attract a growing interest toward the development of small molecules NLRP3 inhibitors. Based on the knowledge of biochemical and structural aspects of NLRP3 activation, one successful strategy in the identification of NLRP3 inhibitors relies on the development of covalent irreversible inhibitors. Covalent inhibitors are reactive electrophilic molecules able to alkylate nucleophiles in the target protein. These inhibitors could ensure good efficacy and prolonged duration of action both *in vitro* and *in vivo*. In spite of these advantages, effects on other signaling pathways, prone to alkylation, may occur. In this review, we will illustrate the chemistry and the biological action of the most studied covalent NLRP3 inhibitors developed so far. A description of what we know about their mechanism of action will address the reader toward a critical understanding of NLRP3 inhibition by electrophilic compounds.

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## References

## 1. Introduction

The nucleotide-binding oligomerization domain (NOD)-like receptor family, pyrin domain-containing 3 (NLRP3 or NALP3) is a cytosolic pattern recognition receptor (PRR) that senses exogenous and endogenous danger signals. Other PRR capable of forming an inflammasome complex are known, among them the most studied are the NLRP1 (NLR family, pyrin domain-containing 1), the NLRC4 (NLR family, CARD domain-containing 4) and the AIM2 (absent in melanoma 2) inflammasomes [1]. The NLRP3 protein is made up of three domains: a leucine-rich repeat domain (LRR), a nucleotide binding oligomerisation domain containing a CARD (caspase activation and recruitment domain) (NACHT), and a pyrin domain (PYD). Upon activation, NLRP3 oligomerises and triggers the helical fibrillar assembly of the adapter apoptosis-associated speck-like protein containing a CARD (ASC) via PYD–PYD interactions. ASC fibrils assemble into large structures, called ASC specks, and recruit pro-caspase-1, leading to its autoproteolytic activation. The activated caspase-1 is able to cleave pro-interleukin(IL)-1 $\beta$  and pro-IL-18 to generate the inflammatory cytokines IL-1 $\beta$  and IL-18 [1,2]. Moreover, activated caspase-1 is also able to cleave gasdermin-D (GSDMD) to its active form (GSDMD-N), depending on cell type, thus triggering programmed cell death, called pyroptosis [3].

The assembled NLRP3 inflammasome plays a key role in the innate immune system. While acute inflammatory conditions are a physiological response to host infection and cellular damage, chronic inflammation is detrimental and can cause secondary damage to cells and host tissues. NLRP3 inflammasome component overexpression and/or NLRP3 inflammasome over-activation have been reported as occurring in many pathological conditions. NLRP3 inflammasome complexes have been identified in several human peripheral and brain tissues, in both *in vivo* and *ex vivo* work. A review that explores inflammasome expression, distribution and activation in a variety of human inflammasomopathies (i.e. autoinflammatory disease caused by inflammasome activity disruption) has been recently published [4].

Gain-of-function mutations in the *nlrp3* gene have been associated with autoinflammatory diseases, including cryopyrinopathies (Cryopyrin-associated periodic syndromes; CAPS), which are a case in point [5-8]. CAPS are caused by an autosomal dominant mutation in *NLRP3* [9]. The term CAPS encompasses three diseases: familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID), the latter of which is also known as chronic infantile neurologic cutaneous articular (CINCA) syndrome [10-12]. These auto-inflammatory diseases generally display neonatal onset and cause a large spectrum of symptoms, ranging from periodic fevers and altered joint/skeletal development to neurological disorders [13].

Observations that uric acid crystals activate the NLRP3 inflammasome led to gout being the first chronic NLRP3-driven inflammatory disorder investigated. While that study was performed by Tschopp's group back in 2006 [14], other pathologies have since been associated with NLRP3 over-activation. Increased NLRP3 response, followed by IL-1 $\beta$  release (and in some cases pyroptotic cell death), has been reported in chronic inflammatory and autoimmune diseases, such as inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) [15-17], non-alcoholic steatohepatitis (NASH) [18], psoriasis [19], autoimmune encephalomyelitis and multiple sclerosis [20].

The pro-inflammatory role played by the NLRP3 inflammasome has also been demonstrated in various rheumatic diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic-onset juvenile idiopathic arthritis (s-JIA), adult-onset Still's disease (AOSD) and crystal arthropathies, such as pseudogout [21-23].

The overexpression of NLRP3 and the activation of the NLRP3-mediated inflammatory response has been observed in recent years in several neurodegenerative diseases [24,25], such as amyotrophic lateral sclerosis (ALS) [26], Alzheimer's disease [27-29] and Parkinson's disease [30-31].

Metabolic and vascular diseases, such as Type 2 diabetes (T2D) and atherosclerosis, have also been linked to NLRP3 overactivation [32-36]. The therapeutic value of NLRP3 inhibition - the dampening of inflammatory responses in animal models of myocardial infarction using a variety of experimental NLRP3 inhibitors - has been demonstrated in several studies [37].

It is worth noting that the beneficial outcomes of blocking IL-1 $\beta$  effects in cardiovascular diseases have recently been demonstrated in the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), which is a clinical trial that has enrolled 10,061 patients with inflammatory atherosclerosis and a prior history of heart attacks [38]. A seminal paper by Latz's group has very recently linked western diet-promoted NLRP3 inflammasome activation to the epigenomic reprogramming of myeloid progenitor cells. This finding opens up new possibilities as to the complex role played by the NLRP3 inflammasome in a number of metabolic and immune-mediated diseases [39-42].

Finally, the NLRP3 inflammasome, together with other inflammasomes, influences the pathogenesis of cancer by modulating innate and adaptive immune responses, cell death, proliferation and/or the gut microbiota. Excessive inflammation, driven by the inflammasome or the IL-1 $\beta$  signalling pathways, promotes breast cancer, fibrosarcoma, gastric carcinoma and lung metastasis in a context-dependent manner [43,44]. These observations have been further corroborated by CANTOS trial findings, which also observed 67 % reduction in lung cancer incidence and 77 % reduction in death from lung cancer in canakinumab-treated patients [45]. The relevance of inflammasomes in multiple forms of cancer might hold promise for the development of a new class of antitumor and anti-metastatic agents that can act on new molecular targets.

Pharmacological agents that function as IL-1 $\beta$  blocking drugs have already been developed. Anakinra, canakinumab, and rilonacept are clinically available biological IL-1 $\beta$  blockers acting through different mechanisms of action. Although these drugs are quite effective, they have several drawbacks: (i) they only target IL-1 $\beta$  response and have no influence on IL-18-mediated effects or pyroptotic cell death-driven inflammation; (ii) they are peptides, meaning that their



pharmacokinetics do not allow for oral administration or blood-brain barrier penetration, thus limiting their use to the treatment of peripheral diseases; (iii) they might increase the risk of opportunistic infections; (iv) they are expensive. The development of small molecule NLRP3 inhibitors would therefore be preferable and provide us with the possibility of developing drugs that can inhibit all NLRP3-mediated responses, be administered orally, and that are cheaper than biological IL-1 $\beta$  blockers. In light of the above, it is not surprising that interest in the NLRP3 inflammasome as a drug target is growing rapidly and medicinal chemists have begun to study the possibility of inhibiting the NLRP3 inflammasome as a new treatment strategy. While specifically designed NLRP3 inhibitors have yet to reach the drug market, a number of small molecule inhibitors of the NLRP3 inflammasome have been developed [46,47].

This review will focus on the medicinal chemistry of covalent NLRP3 inhibitors.

## **2. An overview of NLRP3 inflammasome activation and signalling**

The NLRP3 inflammasome molecular mode of activation remains to be fully defined, however, recent studies have greatly enhanced our understanding of the mechanism. The mechanisms underlying NLRP3 inflammasome activation and signalling are reviewed elsewhere in this issue, and will therefore only be briefly summarized herein (Figure 1) with the scope of illustrating the point of intervention of various NLRP3 blockers that are the object of this review.

NLRP3 inflammasome works as a highly sensitive, but non-specific, surveillance mechanism that responds to any type of perturbation that breaches plasma membrane integrity and the associated K<sup>+</sup> gradient across the membrane [48-51]. As the NLRP3 inflammasome is an important regulation checkpoint for innate immune defence, its activation usually requires two different signals in order to prevent superfluous inflammation, which can be deleterious for the host. These signals are generally referred to as the priming signal (signal 1) and the activation signal (signal 2). However, while two signals are necessary in dendritic cells and macrophages, only one signal is sufficient for NLRP3 activation in monocytes, which, in fact, need to be highly reactive. An alternative

inflammasome activation can occur in monocytes and can lead to the release of inflammatory IL-1 $\beta$  without triggering pyroptosis [48].

During the priming step transcriptional regulation of gene expression is promoted leading to the increase of NLRP3 and pro-IL-1 $\beta$  levels in the cell. Instead, transcriptional regulation is not required for ASC, procaspase-1 and pro-IL-18, which are available in sufficient amount in cell cytoplasm in basal conditions [52]. The crucial up-regulator for NLRP3 and pro-IL-1 $\beta$  is the transcription factor NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells), which is activated upstream via the stimulation of numerous cytokine receptors, such as Toll-like receptors (TLRs), IL-1 receptor 1 (IL-1R1), NOD-like receptors (NLRs), tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2). Signalling from cytokines receptors proceeds through either myeloid differentiation primary response 88 (MyD88) or TIR (Toll/IL-1 receptor) domain-containing adapter-inducing interferon- $\beta$  (TRIF), followed by certain IL-1R-associated kinase (IRAK) family members. IRAK1 and IRAK4 seem to be specifically responsible for activation of inhibitor of NF- $\kappa$ B kinase (IKK) and downstream NF- $\kappa$ B phosphorylation. This signal is essential for NF- $\kappa$ B migration into the nucleus and transcriptional activation to occur.

Once expressed, free cytosolic NLRP3 is maintained in an inactive status thanks to the ubiquitination of its LRR domain, therefore, deubiquitinylation is required prior to its activation.

Inhibition of deubiquitinating (DUB) activity of BRCC3 (JAMM domain-containing Zn<sup>2+</sup> metalloprotease DUBs) inhibits the subsequent activation of NLRP3 [53]. Recent studies have reported that host-derived factors may prime the inflammasome during sterile inflammatory diseases, such as atherosclerosis, metabolic diseases and neuroinflammatory disorders [54].

The activation step is elicited by several stimuli, ranging from exogenous dangers (i.e. bacterial pathogens and pore-forming toxins) to endogenous molecules, such as crystals, aberrant proteins (e.g. amyloid- $\beta$  peptide or  $\alpha$ -synuclein aggregates), and ATP, the latter acting via interaction with the P2X7 receptor. Considering the diversity among these activation signals, several studies have been conducted to identify common downstream effector(s) for NLRP3 activation. The main mechanisms

for NLRP3 activation include: potassium efflux, ROS production, lysosomal damage, and altered calcium signalling [52]. The role of volume-regulated anion channels (VRAC)-mediated chloride efflux in NLRP3 activation has recently been demonstrated in an investigation that worked with fenamate derivatives in both immortalized bone-marrow derived macrophages (iBMDMs) and THP-1 cells [55]. As a matter of fact, a reduction of intracellular  $K^+$  concentration is sufficient to activate the NLRP3 inflammasome [50]. Potassium efflux has been proposed as a common trigger for NLRP3 activation in response to different stimuli. Recent studies report the involvement of NEK7, a Ser/Thr kinase involved in mitotic cell division, in the sensing of low intracellular  $K^+$  [56]. NLRP3 associates with the catalytic domain of NEK7 in a  $K^+$ -efflux dependent manner, to form a high-molecular-mass NLRP3-NEK7 complex, which triggers ASC oligomerization [57]. NEK7 might also have a role in mutant NLRP3 found in CAPS where some mutations can promote binding between NEK7 and NLRP3 [58]. Other activating stimuli, such as bacterial pore-forming toxins, nigericin, ATP, particulate matter, ROS, lysosomal damage, among others, cause  $K^+$  efflux, mitochondrial perturbation and/or the opening of large membrane pores [51].

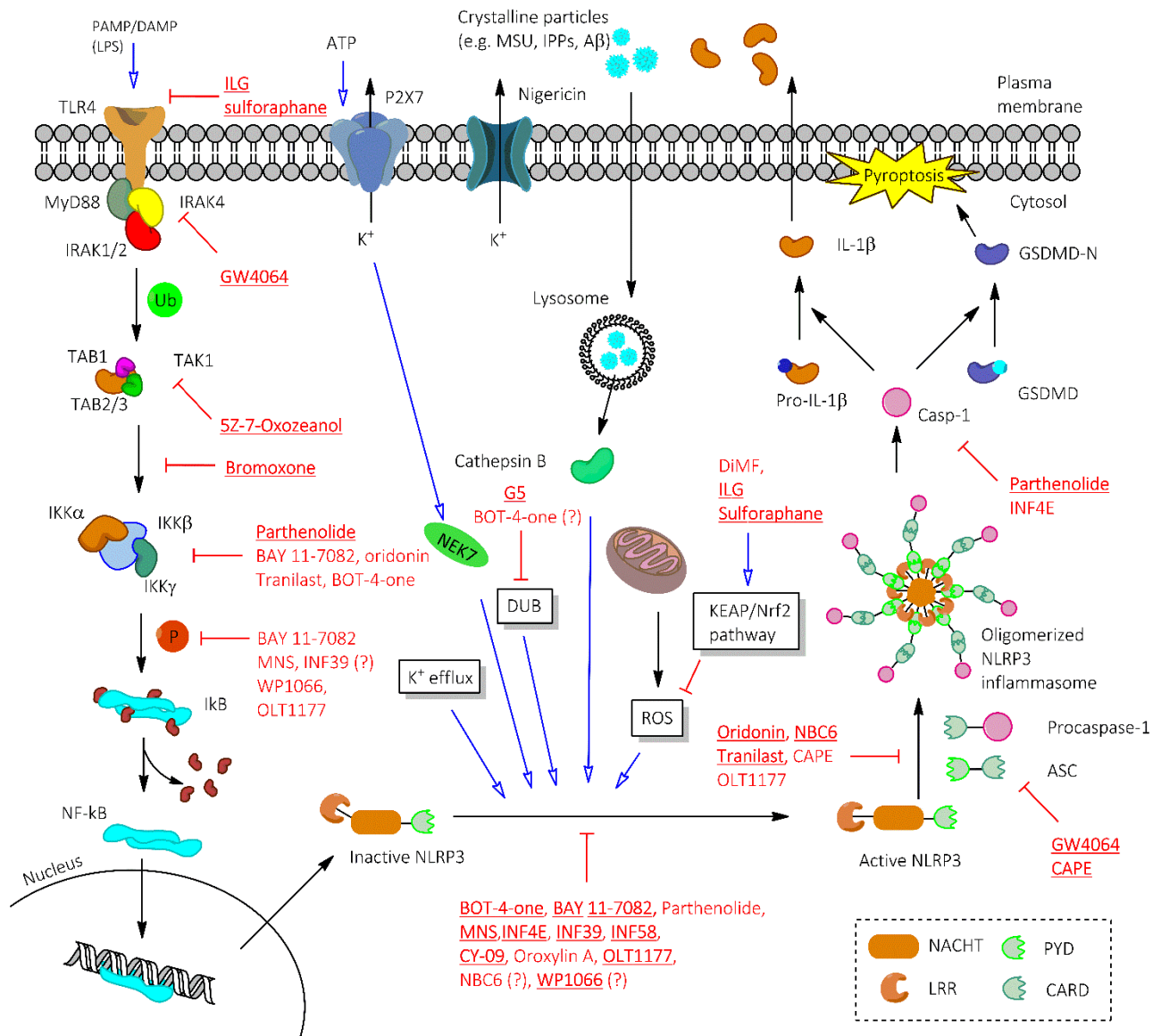
After exposure to a danger signal, presumably recognized via LRR domain, NLRP3 monomers induce self-oligomerisation through the PYD-PYD interactions of ASC. Activated NLRP3-ASC specks recruits and oligomerises procaspase-1, which, activated by proximity-induced auto-cleavage, triggers proteolytic maturation of pro-IL-1 $\beta$  and pro-IL-18 [48,59,60].

Other routes for the activation of the NLRP3 inflammasome, namely non-canonical and alternative inflammasome activation have been recently proposed [61]. Non-canonical NLRP3 inflammasome activation is strictly correlated with gram-negative infection or *in vitro* lipopolysaccharide (LPS)-stimulation. LPS is a ligand of the TLR4 receptor, which triggers NF- $\kappa$ B-mediated NLRP3 and pro-IL-1 $\beta$  expression. Studies on TLR4-deficient mice, showed only partial abrogation of NLRP3 activation in the absence of TLR4-mediated priming activity. This finding suggested that a different kind of activation could be mediated by LPS [62]. Gram-negative bacteria secrete LPS-loaded outer-membrane vesicles (OMVs), delivering LPS into the host cytosol [63,64]. Caspase-11 can be

activated by direct binding of the hexa-acylated lipid A moiety of LPS, secreted by certain gram-negative bacteria (*E. coli*, *S. flexneri*, *S. typhimurium*) via its CARD domain. Humans lack the caspase-11, but caspase-4 and caspase-5 are capable to bind intracellular LPS [65,66], thereby promoting non-canonical NLRP3 activation.

The mechanisms of alternative NLRP3 inflammasome activation remain unclear, but evidence has pointed to a  $K^+$ -independent direct activation of NLRP3 under LPS stimulation. This signalling pathway involves TLR4 in LPS-recognition, TRIF, RIPK-1 (receptor-interacting serine/threonine-protein kinase 1), FADD (Fas-associated protein with death domain), while caspase-8 is the final effector that directly activates NLRP3 through a still-unknown mechanism. Moreover, the alternative activation does not require GSDMD as pyroptotic effector [67,68].

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**Figure 1.** A simplified view of the NLRP3 inflammasome signaling pathways. Blue arrows indicate main activation signals. The points of intervention of NLRP3 inhibitors that are described in this review are indicated in red. In the case of multi-target inhibition the most relevant mechanism is indicated with bold inhibitor name; accessory mechanisms of action are indicated in plain text. DAMP (danger-associated molecular patterns), PAMP (pathogen-associated molecular patterns)

### **3. The NLRP3 inflammasome as a drug target: structural biology and conformational changes.**

Whatever stimulus triggers NLRP3 inflammasome activation, conformational changes are required in the switch from the inactive resting conformation to the active structure that, in its turn, can trigger the downstream cascade. An event that promotes an activating change in NLRP3 conformation is potassium efflux. Bioluminescence Resonance Energy Transfer (BRET) studies have confirmed the importance of this step in changing NLRP3 into a shape that is ready for signalling [49,69]. These studies have also provided precious insight into the resting position of NLRP3. NLRP3 forms preassembled complexes, as is confirmed by the close proximity of NLRP3-PYD to NLRP3-LRR before stimulation. These studies highlight the essential nature of the NACHT domain in the formation of resting complexes.

ATPase activity within the NACHT domain is also mandatory for NLRP3 inflammasome activation. NLRP3 actually needs to cleave ATP into ADP in its ATPase pocket in order to achieve the active conformation [70]. NLRP3 ATPase activity is required for oligomerisation, interaction with ASC and IL-1 $\beta$  release in WT-NLRP3, as well as in CAPS-mutated NLRP3. This suggests that blocking ATPase activity might be a feasible strategy with which to target cryopyrinopathies [7, 71].

All NLRP, including NLRP3, belong to the STAND (signal transduction ATPases with numerous domains) subfamily of AAA+ (ATPases associated with diverse cellular activities). Conserved motifs of the STAND family include an ATPase-specific P-loop, a Mg<sup>2+</sup> coordinating loop, as well as Walker A and Walker B motifs [71]. While no crystal structures are available for any of the NACHT domains of the NLRP family, their similarity with APAF-1 (apoptotic protease activating factor-1) and NLRC4 (NLR family CARD domain-containing protein 4), whose structures have recently been solved, allows structural comparisons to be performed [72,73]. Sequence analyses show high conservations in the Walker A, Walker B, Sensor 1, and Sensor 2 motifs (Figure 2). The conserved Walker A motif is characterized by GxxGxGK(S/T), where the lysine residue (K) is crucial for direct interaction with the  $\gamma$ -phosphate of ATP. Walker B presents a conserved acidic residue (Asp or Glu)

in the hhhhDE motif, characterized by two consequent acidic residues, which coordinates  $Mg^{2+}$  and a molecule of water in a process that is necessary for hydrolysis. Sensor 1, characterized by three hydrophobic amino acids followed by two Ser/Thr residues that are followed by Arg, contributes to the perfect interaction between Walker A and Walker B and the correct positioning of the nucleotide. Sensor 2 is marked by the presence of a conserved Lys or Arg residue, which is responsible for nucleotide coordination, hydrolysis and conformational changes between subunits. It is worth highlighting two regions when comparing APAF-1 and NLRP3: a hydrophobic cluster in the N-terminal of NACHT, and a GxP motif in the C-terminal where the proline is likely to play a crucial role in nucleotide stabilisation [71]. Of the numerous interesting van der Waals interactions, Ser325 in APAF-1 and Trp416 in NLRP3 are particularly important as these residues are part of a Cys-rich motif VCWxVCT that is adjacent to the nucleotide binding site, which is crucial for nucleotide recognition [74]. *In silico* studies have demonstrated that the surroundings of the nucleotide binding sites in NLRP3 consist of Gly229, Ile230, Gly231, Lys232, Thr233 and Ile234, that are oriented around  $\alpha$ - and  $\beta$ -phosphates of ADP. All of these considerations and similarities with Ser/Thr kinase pockets might assist in the development of drugs that disrupt binding with ATP. Two types of ATPase inhibitors can be designed: they are generally defined as type 1 (that occupy ATP-binding sites in the active conformation) or type 2 inhibitors (allosteric modulators of sites adjacent to the ATP pocket). Other domains of NLRP3 and its interacting proteins (such as ASC) need to change conformations to start inflammasome signalling. PYD-PYD interactions might also cause relevant conformational changes [75], as might ASC-ASC interactions [76]. The PYD domain might play a role as the formation of a disulphide bridge between Cys8 and Cys108 has been observed in the active conformation of NLRP3 [77]. The structural mechanisms involved in NLRP3 inflammasome formation have recently been reviewed and are still a matter of investigation [78,79].

Finally, post-translational phosphorylation plays a complex role in the regulation of NLRP3 assembly. Protein-kinase A (PKA) is able to phosphorylate Ser295 in the NACHT domain in already assembled inflammasome, thus inducing inflammasome disassembly [80]. Several recognized

CAPS-related mutations are detected in the NACHT domain that are capable of switching off the regulatory phosphorylation of Ser295 [7]. Phosphorylation at Ser5 in the PYD domain is also capable to hamper inflammasome activation by abrogating PYD-PYD interaction [81].

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1 mkmastrckl aryledledv dlkkfkmhle dyppqkgcip lprgqtekad hvdlatlmid
PYD 61 fngeekawam avwifaainr rdlyekakrd epkwgsdnar vsnptvicqe dsieeewmg1
121 leylsrisic kmkkdyrkky rkyvrserfqc iedrnarlge svslnklytr lrlikehrsq
181 qereqellai gkktctespv spikmellfd pddehsepvh tvvfqGAAGI GKTilarkmm
241 ldwasgtlyq drfdylfyih crevslvtqr slgdlimscc pdpnppihki vrkpsRILFL
NACHT 301 mdgfdelqga fdehigplct dwhkaergdi llsslirckl lpeasLLITT Rpvaleklqh
361 lldhPRHVEi lgfseakrke yffkyfsdea qaraafsliq enevlftmcf iplvcwivct
421 glkqqmesgk slaqtskttt avyvfflssl lqprggsqeh glcahlwglc slaadgiwnq
481 kilfeesdlr nhglqkadvs aflrmnlfqk evdcekfysf ihmtfqeffa amyylleek
541 egrtnvpgrs lklpsrdvtv llenygfek gyilfvvrfl fglvnqerts ylekklscki
601 sqqirlellk wievkanakk lqiqlsqlel fycliemqee dfvqramdyf pkieinlstr
661 mdhmvssfci enchrvesls lgflhnmpke eeeeekegrh ldmvqcvlps sshaacshgl
721 vnshltssfc rglfsvlsts qslteldlsd nslgdpqmr vlcetlqhpgc nirrlwlgrc
781 glsheccfdi slvlssnqkl veldlsdnal gdfgirllcv glkhllcnlk klwlvscclt
LRR 841 saccqdlasv lstshsltrl yvgenalgds gvailcekak npqcnlqklg lvnsqgtsvc
901 csalssvlst nqnlthlylr gntlgdkgik llceglhlpd cklqvleldn cnltshccwd
961 lstlltssqs lrklslgnnd lgdlgvmmfc evlkqqsc11 qnlglsemyf nyetksalet
1021 lqeekpeltv vfepsw

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**Figure 2.** Human NLRP3 sequence ([www.uniprot.org/uniprot/Q96P20](http://www.uniprot.org/uniprot/Q96P20)). Walker A (227-234), Walker B (296-302), Sensor 1 (346-351), and Sensor 2 (365-368) in the NACHT domain (red) are reported in bold capital letters.

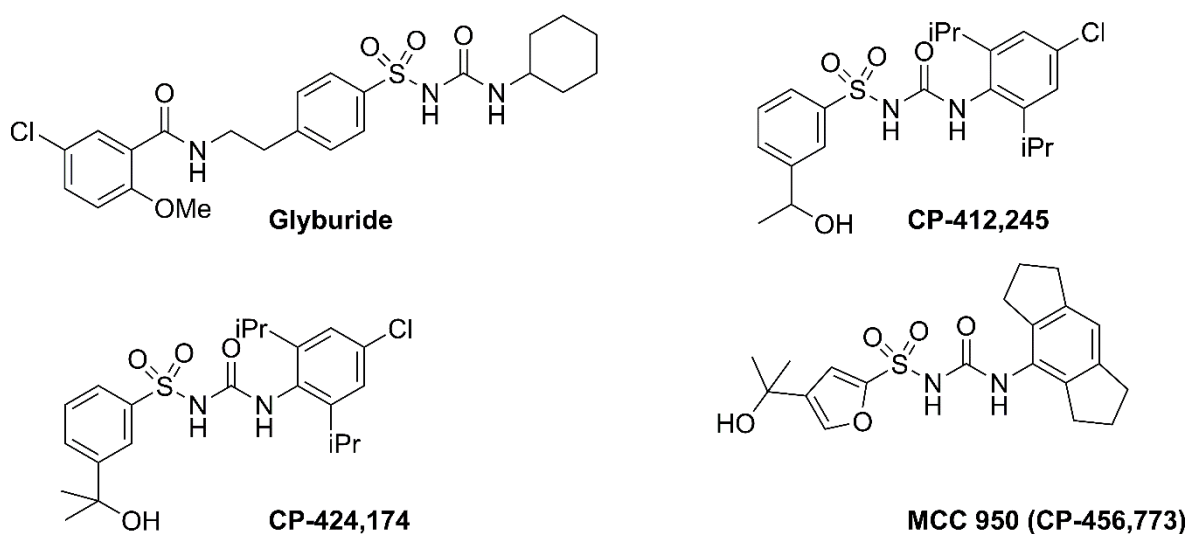
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#### 4. Blocking the NLRP3 inflammasome with covalent inhibitors

Experimental work on the identification of molecules that are able to act as NLRP3 inhibitors has been performed since NLRP3 inflammasome's discovery in 2002 by Tschopp's group [82]. It is



interesting to note that, even before it was discovered, scientists at Pfizer had already identified that the approved drug glyburide and other sulfonylurea derivatives (Figure 3) were able to block IL-1 $\beta$  release in human monocytes following LPS/ATP stimulation [83]. Glyburide and sulfonylurea derivatives that share the ability to block IL-1 $\beta$  release, collectively named CRIDs (cytokine release inhibitory drugs), have been the object of extensive research [84,85], which has led to the selection of MCC950, also known as CRID3 or CP-456,773 (Figure 3), as a potent NLRP3 blocker [86].

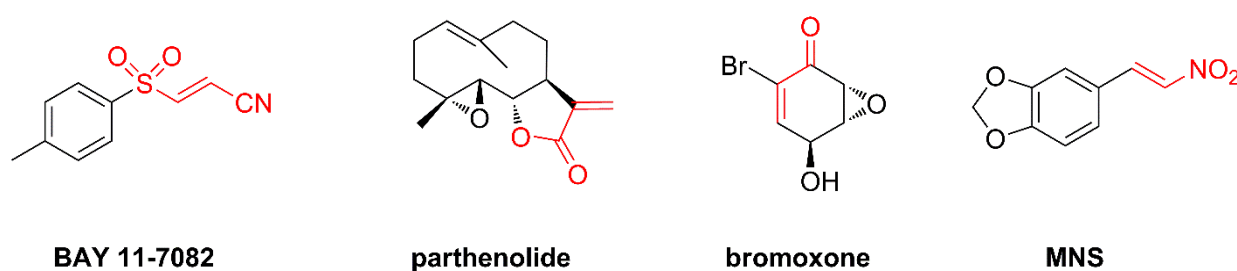


**Figure 3.** Structure of Cytokine release inhibitory drugs (CRIDs)

Two papers, published in 2010, demonstrated that the synthetic small molecule BAY 11-7082, parthenolide, a natural sesquiterpene lactone isolated from *Tanacetum parthenium*, and bromoxone, a natural product isolated from a species of acorn worms [87] (Figure 4), were able to prevent NLRP3-dependent pyroptosis and to inhibit the release of IL-1 $\beta$  from phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells after LPS/ATP stimulation [88,89]. In 2014, He *et al.* published a paper showing that 3,4-methylenedioxy- $\beta$ -nitrostyrene (MNS; Figure 4), a synthetic

kinase inhibitor, was able to block NLRP3 assembly via the direct targeting of NLRP3 ATPase activity [90].

As reported above, most of the early discoveries identified structurally unrelated compounds as direct/indirect NLRP3 inhibitors. With the notable exception of CRIDs, they are all electrophilic derivatives that bear a Michael acceptor moiety in their structure (Figure 4).



**Figure 4.** Structures of electrophilic NLRP3 inhibitors BAY-11708, parthenolide, bromoxone, and MNS. The Michael acceptor moiety is written in red.

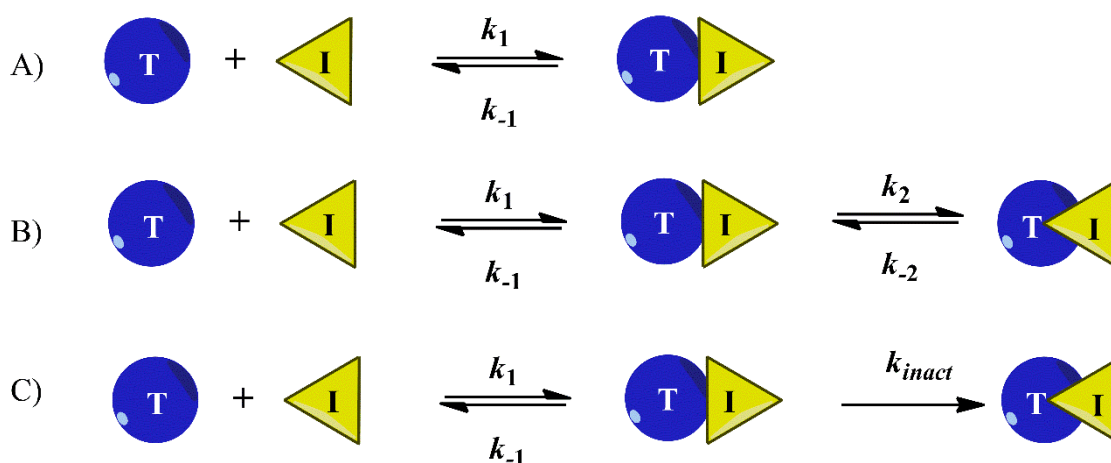
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Michael acceptors are able to react with the biological nucleophilic residues, such as cysteine, serine, lysine and histidine, found in proteins and thus form covalent bonds. These observations allow us to hypothesise that the NLRP3 inflammasome might be a sensible target for the design of targeted covalent inhibitors.

The design of covalent inhibitors has recently gained renewed interest following the approval of kinase inhibitors, including afatinib, ibrutinib and many other marketed drugs [91-93].

The binding kinetics of covalent inhibitors are different to those of traditional non-covalent drugs (Figure 5). While non-covalent inhibitor efficacy and potency correlates with their affinity for the target ( $k_i$ ), the efficacy and potency of covalent inhibitors mainly depends on their residence time ( $t_r$ ), namely the time the drug remains bound to its target. Covalent inhibitors are generally classified into two broad categories, according to whether their adducts with the protein targets are

functionally reversible on a biological time scale. Covalent reversible inhibitors covalently bind to their target protein and subsequently dissociate from it at a rate that is faster than the turnover rate of the protein. On the other hand, covalent irreversible inhibitors form adducts to their protein targets that either do not dissociate from the protein during its lifetime or do so with a kinetic half-life that is significantly longer than the resynthesis rate of the protein. Covalent irreversible inhibitors often contain hard electrophilic moieties, such as epoxides, aziridines and  $\alpha$ -haloketones, in their molecular structure, but most commonly employ Michael acceptors as the electrophilic warhead responsible for the binding to the desired target.



**Figure 5.** The different binding modes of an inhibitor (I) to a protein target (T). A) One-step reversible inhibition; B) induced-fit or covalent reversible inhibition; C) covalent irreversible inhibition.

Covalent inhibitors offer several advantages over non-covalent inhibitors: (i) high biochemical efficiency, which often translates into lower doses and reduced possibility of off-target effects; (ii) non-equilibrium binding, thus overcoming competition from endogenous ligands; (iii) reduced resistance, due to possibly conserved covalent binding to mutated sites; (iv) the uncoupling of

pharmacokinetics/pharmacodynamics and a prolonged duration of action that can result in less-frequent dosing; (v) the ability to address shallow binding sites. Of course, there are also some potential risks involved in developing covalent inhibitors: (i) potential idiosyncratic reactions; (ii) excessive reactivity can lead to drug-induced toxicity; (iii) there are few advantages for proteins that are rapidly turned over; (iv) non-suitability for the target requiring transient inhibition.

The full NLRP3 protein is made of 1036 aa (118.2 kDa) and contains 45 cysteine residues (Figure 2) [94]. NLRP3's 3D structure has not yet been elucidated. However, its close homology with APAF1 and NLRC4 has allowed a homology model of its ATP-binding site to be generated [95].

Apart from the roles of Cys8 and Cys108 in the PYD domain, which appear to be involved in disulphide bond formation upon NLRP3 activation [77], little is known about the role of other 43 Cys residues. Interestingly some cysteines are located in close proximity to the putative ATP-binding pocket [71]. Collectively, these observations have prompted medicinal chemists and biologists to work on covalent NLRP3 inhibitors, which may be able to bind to nucleophilic cysteine residues and exert either the reversible or irreversible inhibition of NLRP3 conformational changes and subsequent activation. The structural properties of the electrophilic warheads help to regulate reactivity and the strength and stability of the covalent bond formed, thereby regulating the residence time of the inhibitor and the reversibility or irreversibility of the action.

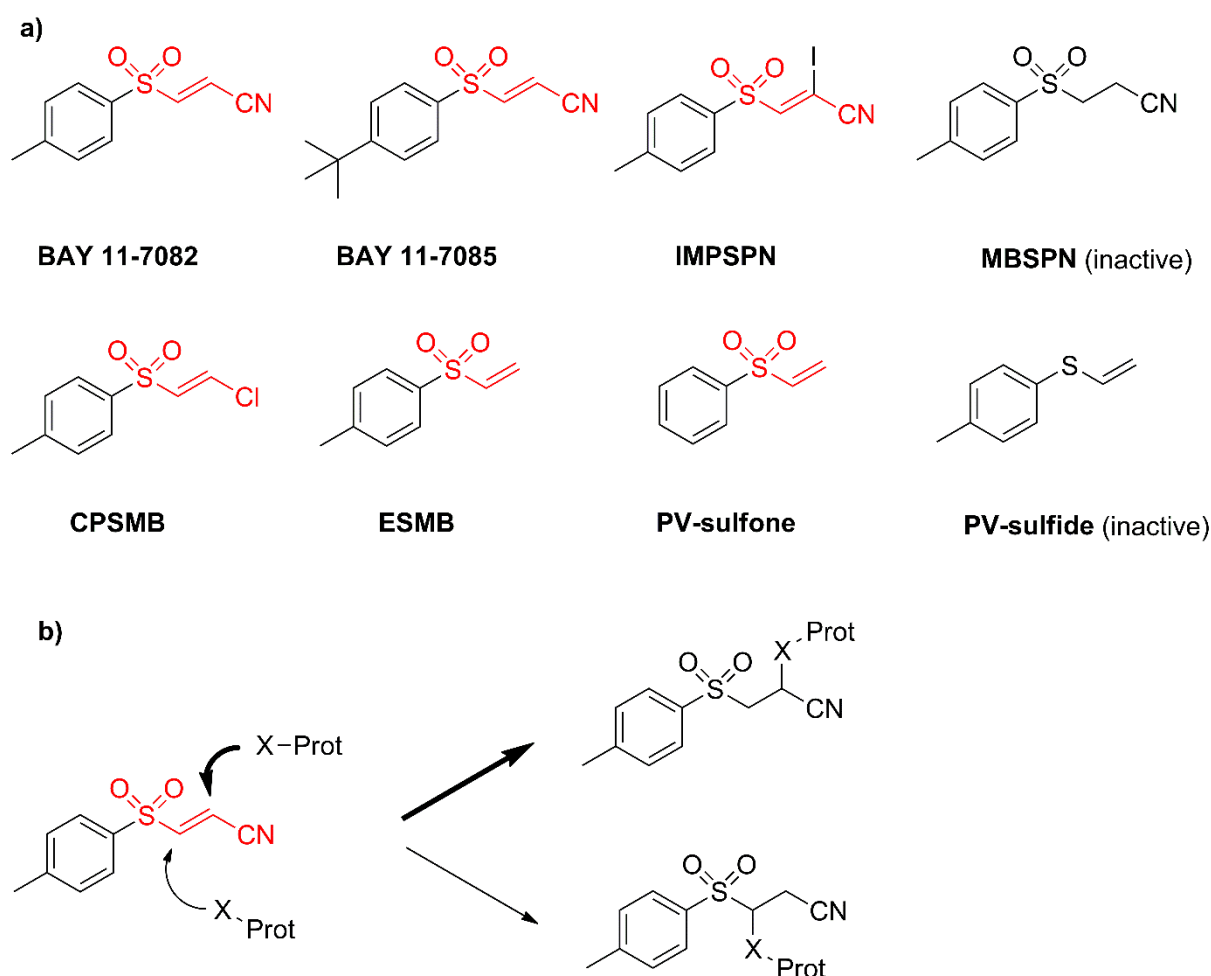
In this review, we describe the chemistry and biological action of NLRP3 inhibitors, that either have been demonstrated to covalently/irreversibly bind to NLRP3 or whose covalent behaviour has not yet been demonstrated, but can be inferred from chemical structure, reactivity and biological activity.

## **5. (E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile (BAY 11-7082)**

BAY 11-7082 (Figures 4, 6a), is a vinyl sulfone that was first synthesised in 1968 [96]. Initially, BAY 11-7082 was thought to inhibit the signal-induced nuclear translocation of NF- $\kappa$ B by inhibiting IKK activity and the phosphorylation of I $\kappa$ B in response to the signal [97,98]. It was later

shown to display its anti-inflammatory action through interactions with multiple targets [99,100], including a series of protein tyrosine phosphatases (PTPs) that act upstream of IKK and are involved in the phosphorylation of the I $\kappa$ B (inhibitor of kappa B) kinase to its active form. BAY 11-7082's mechanism of action relies on its ability to covalently and irreversibly bind to the conserved nucleophilic Cys215 present in the active sites of various PTPs, as has been demonstrated for PTPB1 [101]. In 2010, Juliana *et al.* demonstrated that BAY 11-7082 was able to directly target the NLRP3 inflammasome and inhibit the production of the pro-inflammatory cytokines, IL-1 $\beta$  and IL-18, in a NF- $\kappa$ B-independent manner in macrophages [88]. The authors compared the effects of BAY 11-7082 to those of parthenolide, to those of a series of structurally related vinylsulfones (BAY 11-7085, IMPSPN, CPSMB, ESMB, PV-sulfone), and to those of one sulfonylpropanenitrile (MBSPN) and one sulfide (PV-sulfide) that are derivatives deprived of Michael acceptor ability (Figure 6a).

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**Figure 6.** a) Structures of BAY 11-7082 and related derivatives used for the identification of the pharmacophore. b) Postulated BAY 11-7082 mechanism of reaction with nucleophile (X)-containing proteins. The Michael acceptor domain is highlighted in red.

This study demonstrated that, while parthenolide is a non-selective inhibitor, BAY 11-7082 was able to inhibit NLRP3 inflammasome, while displaying no (or only partial) inhibition of the NLRP1 and NLRC4 inflammasomes. Experiments conducted in NG5 cells, a stable *Nlrp3*<sup>-/-</sup> bone marrow macrophage cell line that constitutively expresses NLRP3 under the control of a murine stem cell virus promoter, established that BAY 11-7082 was able to inhibit ATP-, nigericin- and sodium monourate (MSU)-induced caspase-1 activation. In NG5 cells, BAY 11-7082 also inhibited ASC-

pyroptosome (a large ASC oligomer) formation after ATP challenge. Unlike parthenolide, BAY 11-7082 did not show the ability to directly block caspase-1 activity in THP-1 cell lysates. The direct inhibition of NLRP3 ATPase activity was demonstrated using autoradiography experiments that were performed by incubating purified NLRP3 with [ $\alpha$ - $^{32}$ P]ATP and monitoring its hydrolysis to [ $\alpha$ - $^{32}$ P]ADP. In this experiment, BAY 11-7082 was able to decrease the NLRP3-dependent hydrolysis of ATP in a dose-dependent manner (5-20  $\mu$ M). As expected, the activity of BAY 11-7082 was abolished by the pre-incubation of NG5 cells with thiol-containing nucleophiles, such as glutathione (GSH) and L-Cys, before treatment with the activation stimulus. This observation is consistent with the postulated alkylation of at least one relevant (functional or regulatory) cysteine residue in the target protein. Finally, BAY 11-7082 was able to dose-dependently (1-20  $\mu$ M) inhibit ATP-induced lactate dehydrogenase (LDH) release, which is used as a marker of NLRP3-dependent pyroptotic cell death. Structure-activity relationships (SAR) demonstrated that the presence of the conjugated vinyl group is essential for the activity of BAY 11-7082 and related vinylsulfones, as proven by the absence of activity shown by poor electrophiles, such as MBSPN and PV-sulfide (Figure 6a).

The authors suggested that the postulated nucleophilic attack should occur at the more highly reactive position 2 ( $\alpha$  to the nitrile moiety). However, an attack at the position 3 cannot be completely ruled out (Figure 6b). The essential pharmacophore was identified as being the vinyl sulfonyl substructure. The reactivity of BAY 11-7082 in pseudo-physiological conditions was measured in comparison to that of parthenolide, MNS, dimethylfumarate and ethyl 2-((2-chlorophenyl)(hydroxy)methylacrylate (INF4E), an electrophilic acrylate derivative; the second order rate constant was determined using the cysteamine chemoassay. BAY 11-7082 was the most reactive compound in these experiments (its kinetics was too fast to be accurately determined), followed by parthenolide ( $k_2 = 47.9 \pm 2.9 \text{ M}^{-1} \text{ s}^{-1}$ ), dimethylfumarate ( $k_2 = 39.01 \pm 0.56 \text{ M}^{-1} \text{ s}^{-1}$ ), MNS ( $k_2 = 2.50 \pm 0.12 \text{ M}^{-1} \text{ s}^{-1}$ ), and INF4E ( $k_2 = 0.866 \pm 0.006 \text{ M}^{-1} \text{ s}^{-1}$ ) [102,103]. BAY 11-7082 was found to inhibit both LPS/ATP- and LPS/nigericin-triggered IL-1 $\beta$  release from bone-marrow

derived macrophages (BMDMs) in a dose dependent manner, completely abolishing the latter at 100  $\mu$ M [102].

BAY 11-7082 has recently been tested at 3 mg/kg (i.p. administration) in an *in vivo* mice model of diabetes (inflammation, obesity and insulin resistance) that was generated using a high fat, high fructose diet [104]. The test compound was able to attenuate the diet-induced increase in NLRP3 expression in the liver and kidneys thanks to its ability to partially inhibit NF- $\kappa$ B signalling. BAY 11-7082 was also able to reduce inflammatory mediators, such as IL-1 $\beta$ , IL-18, and TNF, on both the systemic (plasma) and local (liver and kidneys) levels. BAY 11-7082 normalized the diet-induced alteration of metabolic parameters and improved the signalling of insulin by restoring the IRS-1 (insulin receptor substrate 1)/Akt (serine/threonine kinase)/GSK-3 $\beta$  (glycogen synthase kinase 3 beta) pathway, which is dampened by NLRP3 activation. Finally, its chronic administration limited the diet-induced inflammatory damage to the kidneys and liver on both the morphological and functional levels.

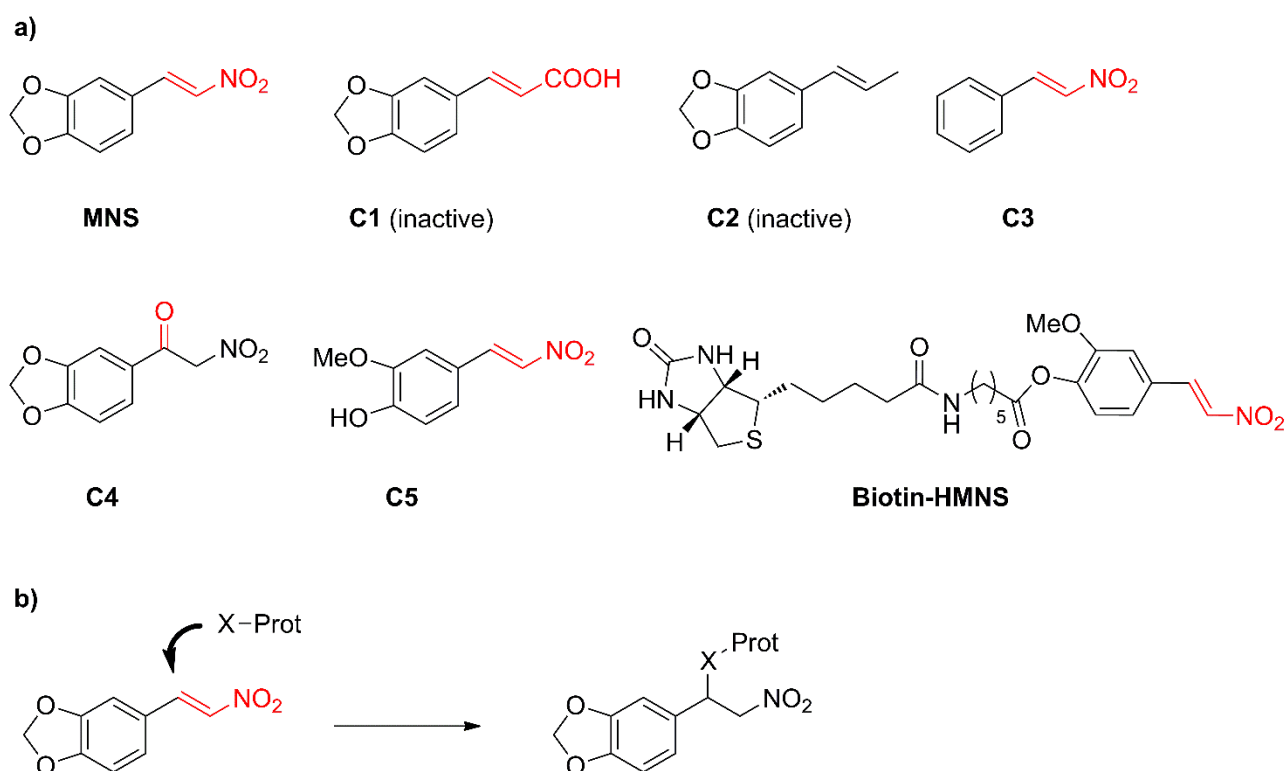
## **6. 3,4-methylenedioxy- $\beta$ -nitrostyrene (MNS)**

MNS (Figures 4, 7a) is a synthetic nitrostyrene derivative, which was originally identified as a Src (proto-oncogene tyrosine-protein kinase) and Syk (Spleen tyrosine kinase) inhibitor that acted at low micromolar concentrations (1-3  $\mu$ M). MNS was characterized for its antiaggregant properties in human platelets and for its ability to irreversibly inhibit p97-containing protein activity [105-107]. In 2014, the screening of 160 kinase inhibitors for their ability to block IL-1 $\beta$  release from LPS-primed/ATP-stimulated BMDMs identified MNS as a NLRP3 inhibitor [90]. MNS was able to dose-dependently (0.5 – 10  $\mu$ M) reduce IL-1 $\beta$  release in this system. IL-1 $\beta$  production was totally blocked at 10  $\mu$ M, while no effect on TNF release was observed. The same results were obtained when the NLRP3 assembly was activated with P2X7 sparing stimuli, such as nigericin and silica. MNS did not have any observable effect on NLRP3, ASC or caspase-1 protein levels, which highlights its lack of action on the NF- $\kappa$ B signalling pathway. In accordance with these results,



MNS proved that it could significantly inhibit LPS/ATP- and LPS/nigericin-induced pyroptotic cell death in BMDMs at 5  $\mu$ M, while it was inactive when cell death was mediated by the activation of NLRC4 or AIM2 inflammasomes, thus displaying its specificity of action against NLRP3. Its activity was largely independent of Syk kinase inhibition, as demonstrated by its ability to work with *Syk*<sup>-/-</sup> macrophages, and was not mediated by interference with K<sup>+</sup> intracellular levels or by direct interaction with caspase-1. Mechanism of action studies suggested that MNS acts directly at NLRP3. MNS dose-dependently inhibited the ATPase activity in recombinant NLRP3 and prevented ASC speck formation in cells. As in the case of BAY 11-7082, a SAR study was conducted using a series of synthetic MNS analogues (Figure 7a). The electrophilic properties of the nitrovinyl moiety were identified as being essential to the biological inhibition of NLRP3 by this study. In the original work, its ability to react with thiols was inferred by it having a reduced effect in the presence of L-Cys. In a later work, using the cysteamine chemoassay it was observed to have marked electrophilic character, with a  $k_2 = 2.50 \pm 0.12 \text{ M}^{-1} \text{ s}^{-1}$  [102]. Moreover, a biotinylated MNS analogue (Biotin-HMNS, Figure 7a), which shared the same ability to inhibit NLRP3, established the direct irreversible binding of MNS to the NOD and LRR domains of NLRP3. Collectively, these studies indicate that the covalent irreversible binding of MNS with cysteine residue(s) (Figure 7b) in the LRR or NACHT domain, which dampens the NLRP3 ATPase activity necessary for protein conformational changes, could be at the basis of its mechanism of action.

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**Figure 7.** a) MNS and derivatives used for pharmacophore identification and in cell immunoblotting studies. b) putative mechanism of nucleophile trapping by MNS.

## 7. Ethyl 2-(2-Chlorobenzyl)acrylate (INF39) and other acrylic acid derivatives (INF compounds)

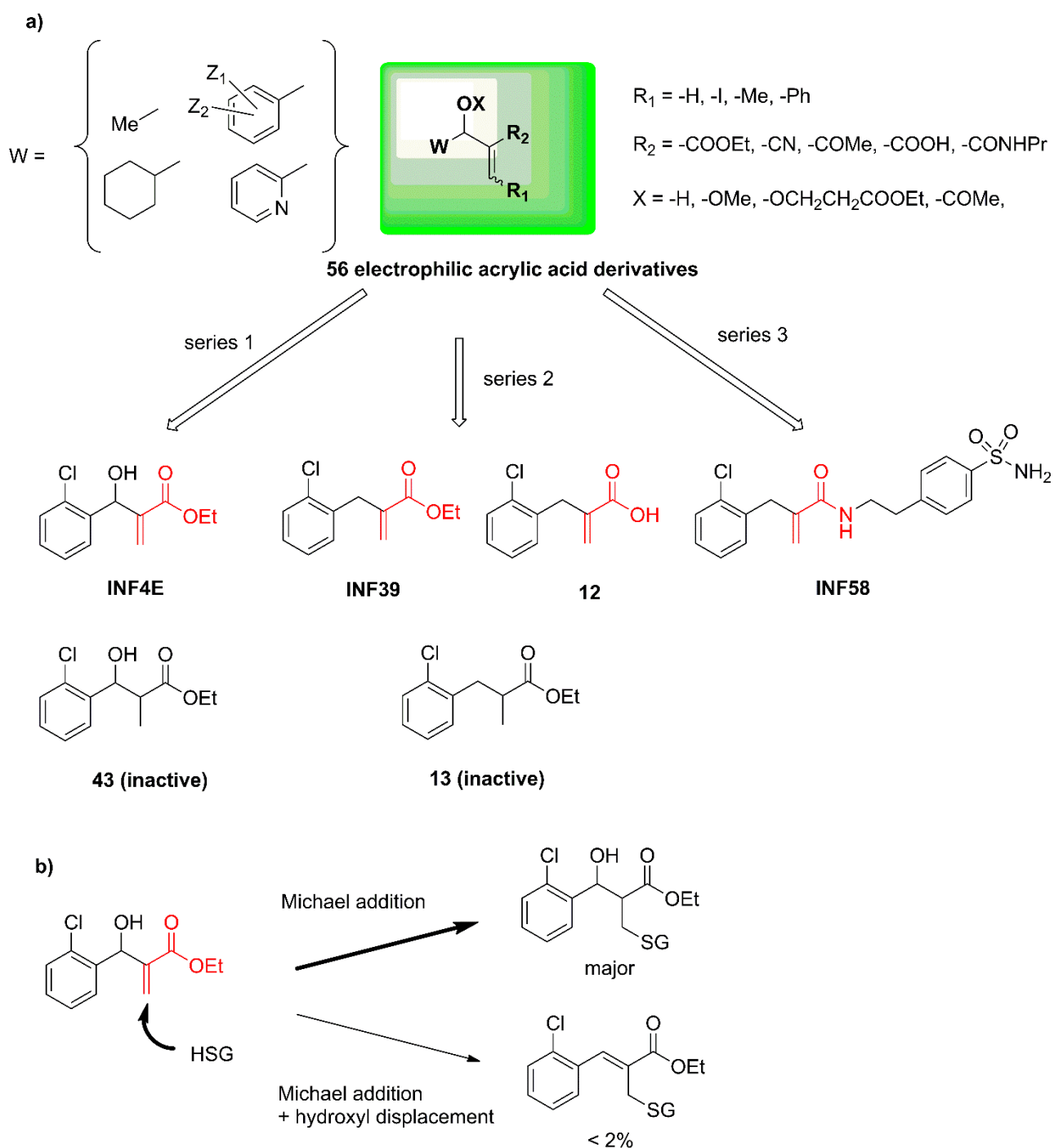
It was observed that compounds containing  $\alpha,\beta$ -unsaturated carbonyl or cyano groups, such as parthenolide, bromoxone, and BAY 11-7082 (Figure 4), were able to directly inhibit the NLRP3 inflammasome, which drove scientists to study the use of electrophilic warheads in the *de novo* design of covalent NLRP3 inhibitors [103]. Cocco et al. have designed a library of differently modulated electrophilic  $\alpha,\beta$ -unsaturated derivatives (Figure 8) and screened their activity in PMA-differentiated THP-1 cells to determine their ability to inhibit NLRP3-dependent pyroptotic cell death [103]. Since electrophilic compounds can also trigger undesired cytotoxicity, the cytotoxic properties of the synthesised compounds were checked in human renal epithelial (HK-2) cells. The

authors identified eleven compounds that were able to prevent > 60 % of LPS/ATP-triggered pyroptosis at 10  $\mu$ M in human macrophages; as expected compounds 13 and 43 (Figure 8a), deprived of the Michael acceptor moiety, were inactive. In particular, INF4E (compound 9 in the cited study) was able to hamper pyroptosis by 75 % at 10  $\mu$ M and was endowed with an acceptable  $TC_{50}$  of 67  $\mu$ M. The acrylate derivatives were found to be generally less cytotoxic than the corresponding acrylonitriles in this work. Further studies, performed using INF4E, confirmed its ability to act as a Michael acceptor and react with GSH to generate a covalent irreversible bond, as demonstrated by NMR studies [103]. As is expected for a covalent irreversible drug, the antipyroptotic effect of INF4E (and of related compounds) was dependent on the pre-incubation time, and reached a plateau after 60 min of cell exposure to the compound prior to the NLRP3-activating stimulus. Dose-dependent reductions of both ATP- and nigericin-induced pyroptosis were obtained, at this pre-incubation time, with  $EC_{50}$  values of 0.12  $\mu$ M and 0.16  $\mu$ M, respectively. A positive correlation was demonstrated to exist between reactivity with cysteamine, measured as a second order rate-constant ( $k_2$ ), and antipyroptotic activity within this series of compounds. INF4E inhibited ATPase activity in recombinant human NLRP3 (rhNLRP3) and showed only partial direct and irreversible caspase-1 inhibition ( $k_i = 9.6 \pm 3.3 \mu\text{M}$ ;  $k_{\text{inact}} = 3.2 \pm 1.1 \text{ s}^{-1}$ ) [102,103]. INF4E was tested in an *ex vivo* model of cardiac ischemia/reperfusion injury in which the formation of a NLRP3 complex was observed [108]. In this model, INF4E attenuated the formation of the NLRP3 complex in a time-dependent manner, and displayed a significant reduction in infarct size and LDH release together with an improvement in post-ischemic left ventricular pressure. Moreover, the hearts of the INF4E-pretreated animals displayed a marked improvement in pro-survival RISK (Reperfusion Injury Salvage Kinase) pathway together with an improvement in mitochondrial function.

The tuning of the reactivity of this series of compounds was performed in an attempt to decrease their cytotoxicity. A series of electrophilic compounds, generated via the chemical modulation of hit compound, INF4E, was designed and synthesised [102]. Their reactivity was efficiently

modulated and a set of less reactive non-cytotoxic compounds was obtained. The synthesised compounds were screened for their antipyroptotic activity and cytotoxicity in THP-1 cells, as well as for their ability to inhibit the ATPase activity of isolated rhNLRP3. This screening process suggested that INF39 (compound 11 in the cited study) was a good NLRP3 inhibitor that was devoid of cytotoxic properties and could irreversibly bind to NLRP3. A BRET assay, performed in HEK293 cells that expressed NLRP3 fused to luciferase and yellow fluorescent protein (YFP), revealed that INF39 affects the conformational changes of the target protein that are necessary for NLRP3 activation, independently of K<sup>+</sup> efflux. Furthermore, INF39 was able to reduce IL-1 $\beta$  release in both LPS/ATP- and LPS/nigericin-stimulated BMDMs and to partially inhibit NF- $\kappa$ B signaling with no direct effect on caspase-1.

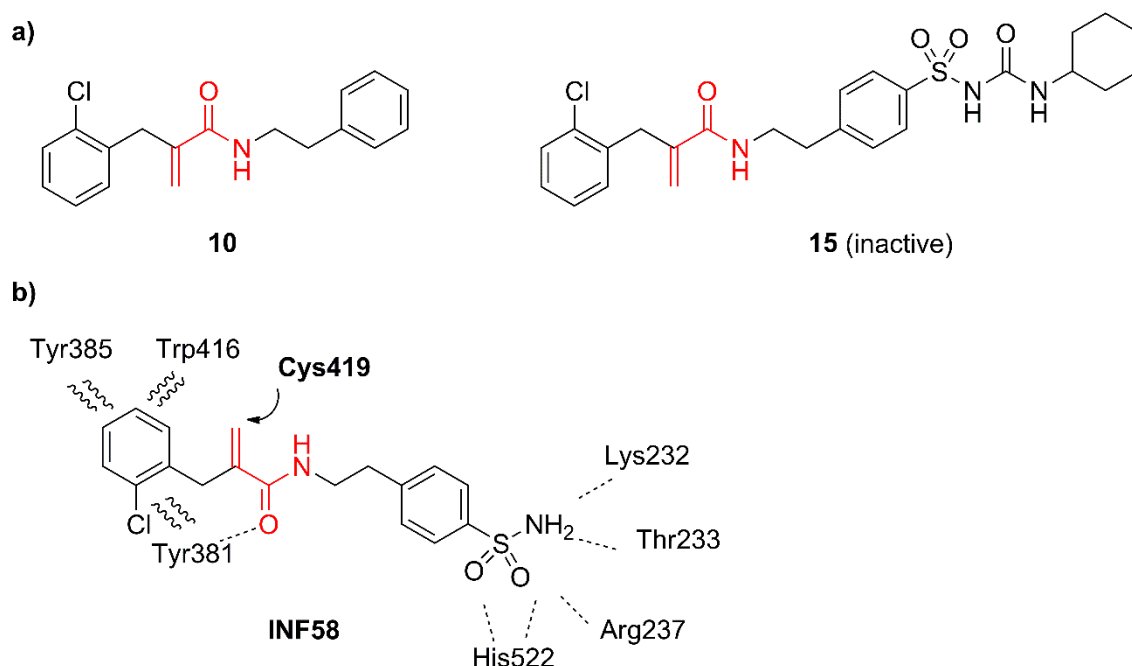
Owing to its reduced reactivity, INF39 was stable in human serum and no detectable binding to human serum albumin (HSA) was observed, unlike its parent INF4E. *In vitro* and *ex vivo* ADME experiments were performed to assess INF39 stability in gastric and intestinal fluids, as well as its ability to be absorbed through the intestinal epithelium. INF39 was stable in the simulated gastric and intestinal fluids, however, it was rapidly metabolized in cells and converted into its active acid metabolite (compound 12, Figure 8). Consequently, INF39 was selected for *in vivo* studies in a rat model of experimental colitis (resembling human Crohn's disease) and the oral route was chosen for *in vivo* experiments. The administration of INF39 in DNBS-treated rats reduced both local and systemic inflammatory markers, such as IL-1 $\beta$ , TNF and tissue myeloperoxidase (MPO), as well as spleen weight. The dose-dependent prevention of colon damage was observed upon morphological and anatomical examination. Overall, INF39 (50 mg/kg) exerted a protective effect that is similar to that shown by the reference drug dexamethasone (1 mg/kg), with no significant lowering of body weight, which is one of the side-effects correlated to chronic administration of steroidal anti-inflammatory drugs.



**Figure 8.** *De novo* design of NLRP3 inhibitors that are based on the acrylic acid scaffold. a) Library of designed electrophilic warheads. Structures of compounds selected from each series are reported. b) Mechanism of reaction of INF4E with glutathione (GSH).

The optimisation of NLRP3-directed electrophilic compounds was also attempted and acrylamide derivatives, which are less reactive than the corresponding acrylates, were used [95, 103]. In this work [95], a series of acrylamides were designed, their reactivity was fine-tuned and selectivity toward NLRP3 was conferred (Figure 8a, 9). Indeed, the acrylamides were found to be less reactive than the corresponding acrylates and no detectable binding to HSA was observed. Compounds 10 (Figure 9) and INF58 (compound 14 in the cited study) were able to prevent NLRP3-dependent pyroptosis with IC<sub>50</sub> values (1 h preincubation) of 12.7 and 23.2  $\mu$ M, respectively, despite being less reactive than INF4E. Both compounds were able to inhibit the ATPase activity of rhNLRP3 and decrease IL-1 $\beta$  release in iBMDMs, primary BMDMs and primary peritoneal mouse macrophages with no effect on TNF release. Interestingly, a molecular hybrid, which was formed by joining the acrylamide function to the glyburide substructure, was found to be inactive in this study (compound 15, Figure 9). Moreover, these acrylamide derivatives also significantly inhibited IL-1 $\beta$  release in macrophages that bear typical mutations (R258W, A350V, L351P), as detected in the CAPS pathology. A homology model of the NLRP3 NACHT domain, which contains the ATP-binding region, was obtained using the resolved structure of NLRC4 (PDB ID: 4KXF), and a putative binding mode for INF58 was proposed (Figure 9) [95]. According to this hypothesis, Cys419 is thought to be the nucleophilic residue that is nearest to the molecule bound in the ATP-binding pocket, thus allowing it to approach and covalently bind to the terminal position of the double bond in the acrylamide pharmacophore.

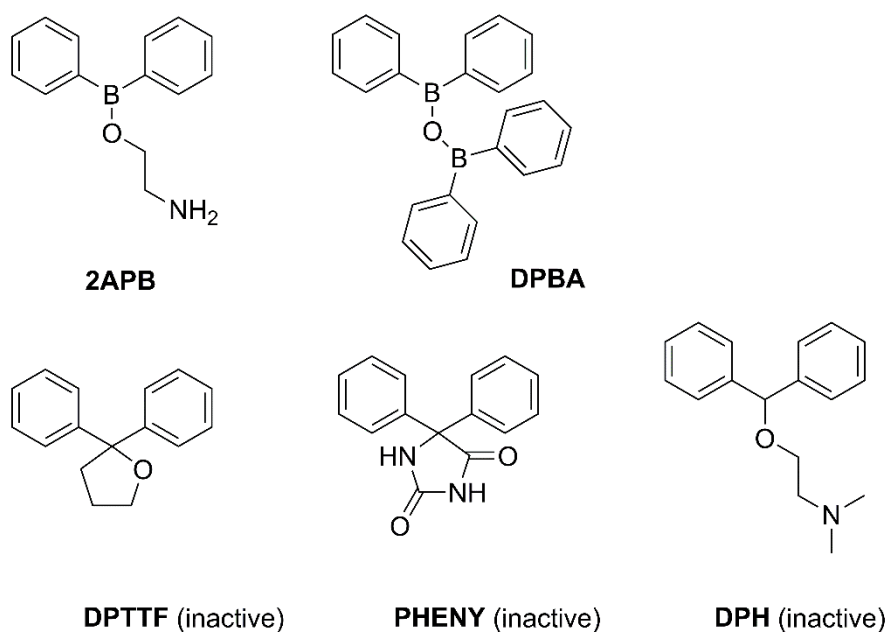
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**Figure 9.** a) Structures of acrylamide derivatives **10**, **15** and **INF58**. b) Putative interactions for **INF58** in the ATP binding pocket.

## 8. 5-Acetyl-6-amino-2,2-diphenyl-4-(trichloromethyl)-2,3-dihydro-1,3,2-oxazaborinin-1-ium-2-uide (NBC6) and related cyclic diaryl boron derivatives

The observation that the  $\text{Ca}^{2+}$  homeostasis inhibitor, 2-aminoethoxy diphenylborinate (2APB; Figure 10), was also able to inhibit the NLRP3 inflammasome [69,109] drove Baldwin *et al.* to develop new boron-based selective NLRP3 inhibitors [110]. After validating the effect of 2APB in LPS-primed peritoneal macrophages, which had been stimulated with different NLRP3-activating agents (nigericin, ATP, MSU, calcium pyrophosphate dehydrate, sphingosine and aluminium hydroxide), a carefully chosen series of diphenyl-substituted compounds (Figure 10), which are structurally similar to 2APB, was tested in order to discover the molecular determinants for the inhibition of NLRP3 inflammasome by 2APB.



**Figure 10.** Representative compounds used for 2APB pharmacophore identification.

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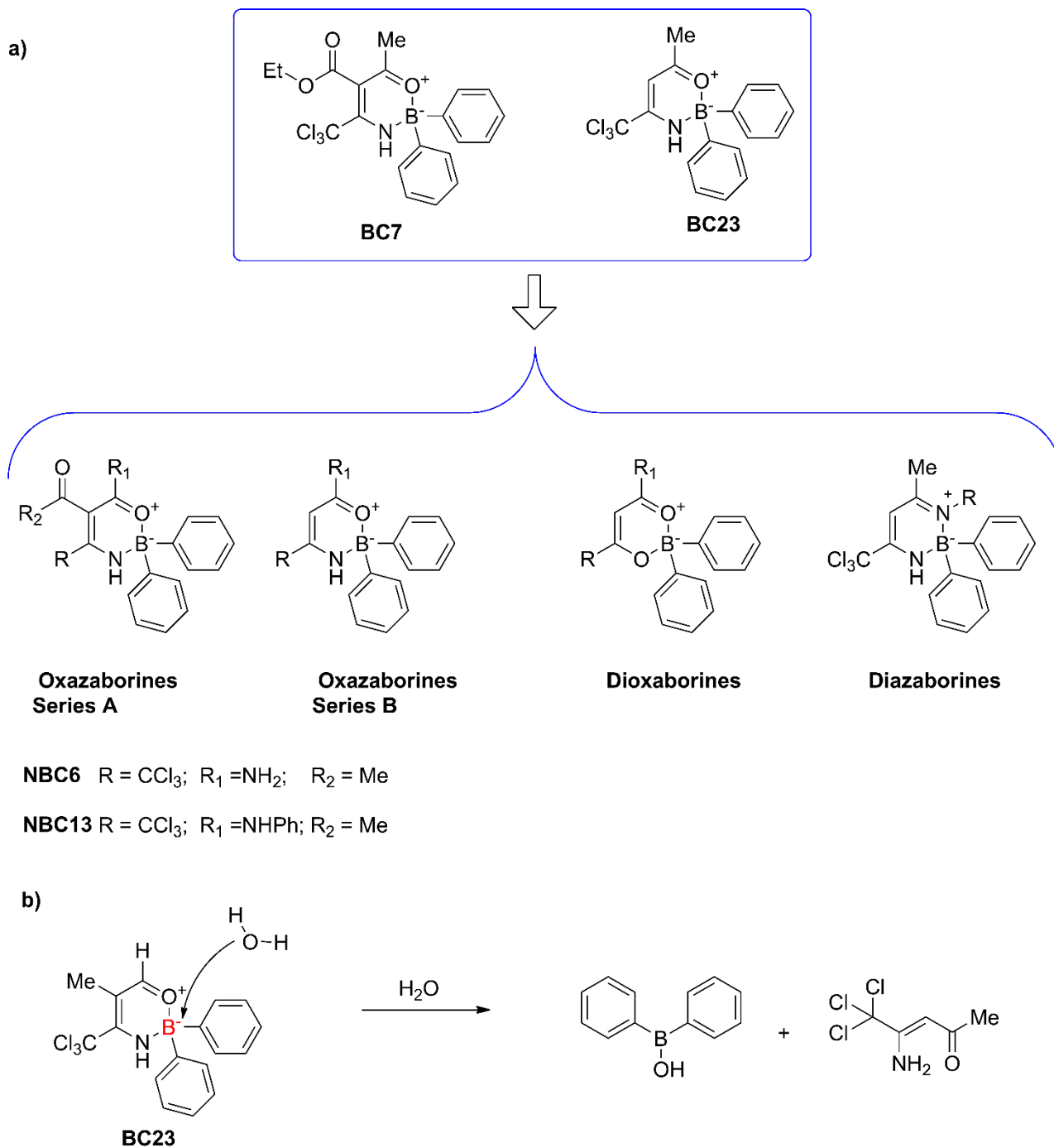
The authors successfully identified that a boron atom linked to an oxygen atom was the minimal pharmacophore necessary for the inhibition of NLRP3-dependent IL-1 $\beta$  release in cells. The synthesis of a series of boron-containing compounds identified two potent hit compounds, BC7 and BC23 (Figure 11), which both bear a diphenyl substituted oxazaborine ring. These compounds displayed IL-1 $\beta$  inhibition with IC<sub>50</sub> values of 1.2 and 2.3  $\mu$ M, respectively in LPS/ATP-stimulated BMDMs. Three series of differently substituted oxazaborines, dioxaborines, and diazaborines (Figure 11a) were further synthesised and screened, which led to the identification of oxazaborine derivative NBC6 as the most promising compound of those investigated. NBC6 inhibited NLRP3-dependent IL-1 $\beta$  release (IC<sub>50</sub> = 0.574  $\mu$ M in LPS/nigericin-treated THP-1) with an action that was not dependent on either intracellular [Ca<sup>2+</sup>] or direct caspase-1 inhibition. SAR were also determined for the borine-derived scaffold, leading to the finding that the electron-withdrawing and lipophilic trichloromethyl substituent at position 4 of the ring played a fundamental role.



Importantly, X-ray studies displayed the electrophilic character of the boron atom present in the oxazaborine derivatives [110,111]. The electrophilic character of these compounds is further supported by the hydrolysis of BC23 observed in DMSO/D<sub>2</sub>O solution at 37 °C; in this condition BC23 displays a half-time of approximately 24 h (Figure 11b) [111]. It is worth noting that NBC6 was able to block both canonical and non-canonical NLRP3 activation, was selective with respect to NLRC4 and absent in melanoma 2 (AIM2) inflammasomes and was devoid of cytotoxic properties in HEK293 and HepG2 cells. In terms of mechanism, NBC6 was able to prevent ASC-speck formation, which is a marker of inflammasome assembly, and irreversibly block NLRP3-dependent IL-1 $\beta$  release from iBMDMs, which is similar to behaviour shown by the nitrostyrene derivative, MNS, that was used as the reference compound in the study. NBC6 also inhibited IL-1 $\beta$  release from neutrophils at 10  $\mu$ M.

NBC13 (Figure 11a), an analogue of NBC6 that shares the same *in vitro* potency (95 % IL-1 $\beta$  release inhibition at 10  $\mu$ M in LPS/nigericin-stimulated THP-1), has been selected for *in vivo* studies in a model of LPS-driven peritonitis. NBC13, administered by oral gavage at a dose of 50 mg/kg, was able to decrease IL-1 $\beta$  levels in peritoneal lavage fluid and those of both IL-1 $\beta$  and IL-1 $\alpha$  in plasma, similarly to the reference compound MCC950. These data suggest that cyclic diarylboron derivatives show promise in their future development as potential drugs for irreversible NLRP3 inhibition [112].

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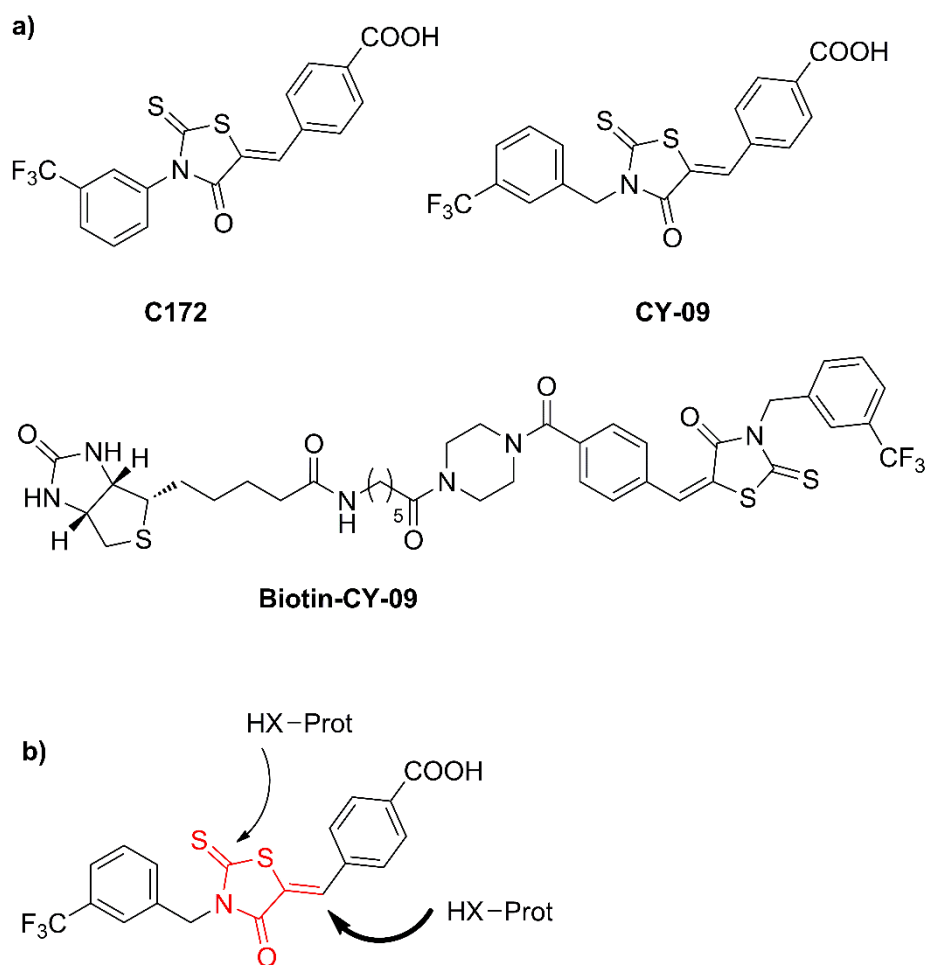
**Figure 11.** a) Development of oxazaborines, dioxaborines, and diazaborines from hit compounds BC7 and BC23; b) nucleophilic attack of water to the boron atom of BC23.

**9. (Z)-4-((4-Oxo-2-thioxo-3-(3-(trifluoromethyl)benzyl)thiazolidin-5-ylidene)methyl)benzoic acid (CY-09)**

In 2017, Jiang *et al.* reported on the discovery of a class of thiazolidinone derivatives that act as potent NLRP3 inhibitors [113]. The first member of this class, namely C172 (Figure 12a), was identified via a screening of a library of cystic fibrosis transmembrane conductance regulator (CFTR) channel inhibitors. The screening, conducted in LPS/nigericin-stimulated BMDMs, revealed that C172 was able to dose-dependently (0.5 – 20  $\mu$ M) reduce caspase-1-mediated IL-1 $\beta$  release with no detectable effect on LPS-priming. Its ability to inhibit CFTR was not responsible for its NLRP3 inhibition, as demonstrated in work with *cfr*<sup>-/-</sup> BMDMs. The authors later tested a series of previously synthesised C172 derivatives [114]. A compound named CY-09 (Figure 12) emerged from this screening as a potent NLRP3 inhibitor with negligible activity on CFTR [113].

CY-09 exhibited a dose-dependent (1–10  $\mu$ M) inhibitory effect on MSU-, nigericin- and ATP-induced caspase-1 activation, and IL-1 $\beta$  secretion in LPS-primed BMDMs. Moreover, CY-09 inhibited non-canonical NLRP3 activation as driven by the administration of intracellular LPS. All these effects were independent of LPS-induced priming and NF- $\kappa$ B activation, as demonstrated by a lack of activity on TNF secretion and on the expression of pro-IL-1 $\beta$  and NLRP3. In keeping with these results, CY-09 was also able to prevent the NLRP3-dependent pyroptosis of BMDMs that were treated with LPS/nigericin by approximately 80 % at 10  $\mu$ M. CY-09 was found to be as potent as BAY 11-7082, parthenolide and sulforaphane, while being less effective than MCC950 in inhibiting IL-1 $\beta$  release from LPS/nigericin-stimulated BMDMs. Its NLRP3 selectivity over NLRC4 and AIM2 inflammasomes was also demonstrated.

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**Figure 12.** a) Structure of hit compound C172, lead compound CY-09 and its biotinylated analogue. b) Possible point of attack by protein nucleophiles on CY-09.

The mechanism of action of CY-09 was also investigated. Treating PMA-differentiated THP-1 cells with a biotinylated analogue (Biotin-CY-09; Figure 12a) demonstrated that CY-09 binds directly to the NACHT domain of NLRP3 with an equilibrium dissociation constant ( $K_D$ ) of 0.500  $\mu\text{M}$ , as measured in thermophoresis experiments. The binding of CY-09 appears to occur at the Walker A motif level and results in the dose-dependent inhibition of NLRP3 ATPase activity, with a maximal 85 % inhibition at 1  $\mu\text{M}$ . This inhibition is highly specific for NLRP3 ATPase with regards to NLRC4, NLRP1, NOD2 and retinoic acid-inducible gene I (RIG-I), as proven by work with

purified proteins. In summary, the authors proved that CY-09 was able to bind to NLRP3, inhibiting its ATPase activity and consequently dampening NLRP3 oligomerisation, NLRP3-ASC recruitment and inflammasome assembly. No effects on potassium efflux, chloride efflux, mitochondrial damage or mitochondrial ROS production were detected.

CY-09 reduced nigericin-triggered caspase-1 activation and IL-1 $\beta$  secretion both in human macrophages and in peripheral blood mononuclear cells (PBMCs) in a dose-dependent manner (1-10  $\mu$ M). The treatment of synovial fluid cells (SFCs), which had been obtained from patients diagnosed with acute and chronic gout, with CY-09 (5 and 10  $\mu$ M) led to a remarkable reduction in activated caspase-1 and IL-1 $\beta$  levels, while having no effect on TNF.

A preliminary *in vitro* pharmacokinetic profile showed that CY-09 has a half-life of 145 min in human and liver microsomes, while it demonstrated no relevant inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6 or CYP3A4. Furthermore, no effects on the human ether-a-go-go (hERG) channel were observed up to 10  $\mu$ M. After a single oral dose (10 mg/kg) of CY-09, a maximal concentration ( $C_{max}$ ) of 3.25 ng/mL was detected in the plasma of C57BL/6j mice after 30 min ( $t_{max}$ ). Bioavailability was 72 %, half-life ( $t_{1/2}$ ) 5.1 h, and the mean residence time  $MRT_{(0-\infty)}$  was 2.4 h.

*In vivo* studies showed that CY-09 displays promising activity in mice bearing the *Nlrp3* (A350V neoR) mutation, which is associated to human MWS-syndrome. In this model, the oral administration of 40 mg/kg CY-09 significantly prolonged the survival time of mutant mice, suggesting that the lethal inflammation caused by the *NLRP3* gain of function mutation was suppressed.

CY-09 was tested in high-fat diet- (HFD) fed mice, which are a model of Type 2 Diabetes (T2D). In this model, the intraperitoneal administration of CY-09 (2.5 mg/kg QD) for six weeks produced beneficial effects for body weight, insulin sensitivity and blood glucose levels in HFD-WT mice, as compared to HFD-*Nlrp3*<sup>-/-</sup> mice, thus underlining the NLRP3-dependent action *in vivo*.

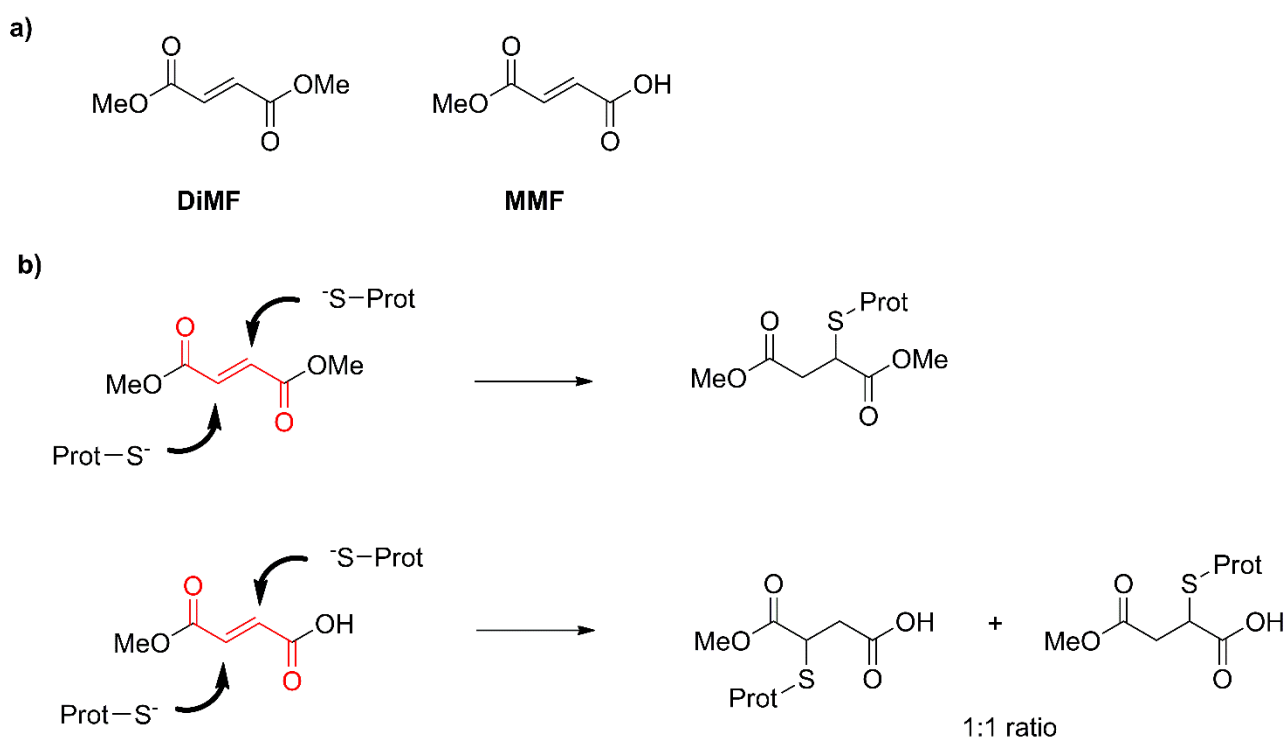
Metaflammation [115] and hyperglycaemia also decreased in HFD-treated mice. Furthermore, CY-09 had no adverse effects in untreated mice that had been fed a standard diet.

Interestingly, the authors did not check for the reversibility of NLRP3 inhibition, in either the isolated protein or in cells, in this work. However, our experience working with covalent inhibitors leads us to believe that, given the electrophilic moiety present in CY-09, the methylene group at the 5 position of the thiazolidinone ring would most likely behave as an electrophile (Figure 12b) and irreversibly/reversibly trap the nucleophilic residues present in the proximity of Walker A motif of the NACHT domain in NLRP3. This behaviour can be also inferred from CY-09's prolonged action in the MWS-mutant mice model, which lasted for a few days (5-23 days) after drug withdrawal. However, the reversibility of this binding in a cellular environment is still to be confirmed. The vast amount of *in vivo* data justifies the development of CY-09 into an effective small molecule NLRP3 inhibitor.

## **10. Dimethyl fumarate (DiMF) and monomethyl fumarate (MMF)**

The first therapeutic use of dimethylfumarate dates back to 1959 when W. Schweckendiek treated himself with a mixture of fumaric acid esters (FAEs) in an attempt to relieve the symptoms of psoriasis [116]. The treatment was somewhat successful and, although the FAEs mechanism of action was not clear, several clinical trials demonstrated the effectiveness and safety of oral treatment with FAEs [117,118]. In 1994, Fumaderm®, a drug product that consists of dimethyl fumarate, calcium-, magnesium- and zinc-salts of monoethyl fumarate was approved in Germany as an oral treatment for *psoriasis vulgaris*. Later clinical research demonstrated that dimethyl fumarate (DiMF; Figure 13a), also labelled as BG-12 in clinical studies, showed beneficial therapeutic effects in patients affected with relapsing-remitting multiple sclerosis (RRMS) [119-121]. Finally, Tecfidera®, a drug product containing DiMF, was approved for the treatment of RRMS in 2013. Although vast amounts of *in vivo* data are available, the DiMF mechanism of action is still not fully understood.

It is important to note that, after oral administration, DiMF is readily hydrolysed, either spontaneously or by esterases, to its metabolite monomethylfumarate (MMF; Figure 13a). No detectable amount of DiMF is present in the portal vein or in plasma, while MMF and the GSH-conjugated derivative (S)-(1,2-dimethoxycarbonyl)ethyl)glutathione (GS-DMS) are readily formed and can be detected both *ex vivo* and *in vivo* [122,123]. Both DiMF and MMF are reactive Michael acceptor molecules and can react with biological nucleophiles (Figure 13b), such as the Cys residues of proteins and GSH, but DiMF shows the greater reactivity due to its neutral nature at pH 7.4, while MMF is monoanionic at the same pH [102, 124].



**Figure 13.** a) Molecular structure of dimethylfumarate (DiMF) and its metabolite monomethylfumarate (MMF); b) Reaction pathways of DiMF and MMF with biological thiols.

In 2015, Miglio *et al.* tested the ability of DiMF and its monomethyl metabolite MMF to inhibit NLRP3-mediated pyroptosis in differentiated THP-1 cells that had been stimulated with LPS/ATP. Both of these compounds were able to irreversibly inhibit pyroptotic cell death and their effects were time- and dose-dependent. Interestingly, both the potency and the efficacy of tested FAEs were related to their reactivity and cell permeability, with DiMF ( $\text{pIC}_{50} = 6.6$ ) being more potent than MMF [125].

DiMF and MMF inhibited IL-1 $\beta$  release and caspase-1 activation in LPS/ATP-treated human macrophages and had no significant direct effect on caspase-1 activity or pro-IL-1 $\beta$  expression. This effect was concentration-dependent, in the 0.01 – 10  $\mu\text{M}$  range, with a determined  $\text{pIC}_{50}$  of 5.8 for DiMF and 6.8 for parthenolide, which was used as the reference comparator in this study. MMF, the most abundant *in vivo* metabolite of DiMF, failed to reach 50 % inhibition, even at 10  $\mu\text{M}$  concentration, in both pyroptosis and IL-1 $\beta$  release experiments. Finally, DiMF did not inhibit NLRP3 ATPase activity in rhNLRP3 [102]. *In vitro* inhibition of NLRP3 activation, triggered with different stimuli (LPS/ATP, LPS/cholesterol and LPS/alum), was recently confirmed for both DiMF and MMF in THP-1 cells, together with their ability to dose-dependently inhibit the formation of intracellular ASC-specks [126]. Until now, no data about the specificity of DiMF or MMF toward NLRP3 inflammasome have been reported.

Recent evidence suggests that MMF may also act via a complex and multitarget mechanism, involving agonism of the hydroxycarboxylic acid 2 (HCA2) receptor and the activation of the Nuclear Factor (erythroid-Derived 2)-Like-2 (Nrf2) pathway via the alkylation of Kelch-like ECH-associated protein 1 (Keap1), which is a physiological effect triggered by several electrophilic molecules [127]. The crosstalk between Nrf2-triggered pathways and NLRP3 signalling is rather complex; as a matter of fact, anti-inflammatory and immunomodulatory effects for DiMF were evidenced in both WT and *Nrf2*<sup>-/-</sup> autoimmune encephalomyelitis (EAE) mouse models [128]. These effects are likely to be mediated by NLRP3 inhibition. Moreover, a recent paper [129] demonstrated that Nrf2-induced gene expression is not involved in inflammasome regulation, while



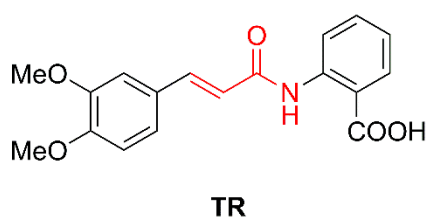
DiMF acts directly on NLRP3 to dampen inflammasome-dependent inflammation in vivo. These two pathways are inversely correlated in cells, where NLRP3 activation downregulates Nrf2 expression. The characterization of electrophilic NLRP3 inhibitors is complicated by the fact that most of the electrophilic compounds are able to interact with Keap1, thereby activating the Nrf2-mediated responses. When working in cells, these two effects can be discriminated by using different experiment times: the NLRP3-mediated pyroptotic cell death is rapidly (< 1 h) triggered by common NLRP3 activating stimuli and inhibited by electrophilic NLRP3 inhibitors, while Nrf2-mediated gene expression usually requires longer times (> 3 h) to be detected [103].

### **11. N-(3',4'-dimethoxycinnamoyl)-anthranilic acid (Tranilast)**

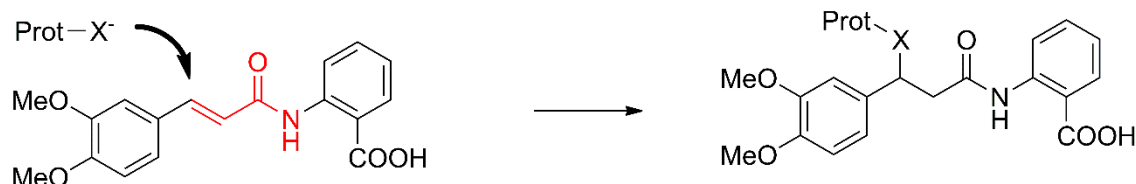
Tranilast (TR; Figure 14a) is an approved drug that is clinically used for the therapy of asthma and other inflammatory diseases in South Korea and Japan. TR is generally well tolerated in patients. However, much like DiMF, its mechanism of action is still poorly understood [130]. Huang *et al.* have recently discovered that TR was able to prevent NLRP3-activation in LPS/nigericin-treated BMDMs by inhibiting inflammasome assembly [131]. TR binds to the NACHT domain of NLRP3 both in cells and in the isolated protein, as demonstrated in pull down experiments conducted with a biotinylated analogue (undisclosed structure). TR proved to be selective for NLRP3 as it was unable to bind to NLRP1, NLRC4, NOD1 and NOD2 in HEK293 cells that overexpress these proteins. This direct effect on inflammasome assembly results in the inhibition of caspase-1 activation and IL-1 $\beta$  secretion both in BMDMs and in human macrophages. These effects were dose-dependent (25 – 100  $\mu$ M) with a maximal inhibition of approximately 90 % at 100  $\mu$ M. Interestingly, TR was able to block the NLRP3-dependent IL-1 $\beta$  release triggered by a variety of NLRP3-activating stimuli, such as ATP, MSU, Alum and cytosolic LPS (cLPS). No effects on either NLRP3 and pro-IL-1 $\beta$  expression, or on IL-6 and TNF production were observed when TR was administered 3 h after the LPS stimulation of BMDMs. However, a decrease in pro-IL-1 $\beta$  expression and IL-6 production was found when TR was administered 30 min before LPS challenge, thus suggesting

that possible interference with NF- $\kappa$ B pathway is present. Effects on NLRP3 expression and TNF production were negligible in the same model. Experiments using cLPS demonstrated that, while TR was able to block non-canonical NLRP3 activation, it was not able to prevent cLPS-induced gasderminD cleavage and subsequent pyroptotic cell death in BMDMs. Finally, the activation of NLRC4 and AIM2 inflammasomes was not blocked, which demonstrates that NLRP3 may be a preferential target for TR. The presence of a direct effect on intracellular  $K^+$  concentration or on mitochondrial damage and ROS production was also excluded.

a)



b)



**Figure 14.** a) Molecular structure of tranilast (TR) electrophilic functionality is written in red; b) possible pathway for covalent interaction with protein target.

Co-immunoprecipitation studies clarified that TR acts by directly binding to NLRP3 and thus prevents NLRP3-NLRP3 and NLRP3-ASC interactions. This mode of action allows the blockade of NLRP3-oligomerisation and the subsequent NLRP3 assembly. Interestingly, TR does not inhibit the ATPase activity of NLRP3, indicating that the binding site is likely to be far away from the ATP-

binding pocket in the NACHT domain of NLRP3. From a mechanistic point of view, it is predictable that TR is able to target a shallow binding site on NLRP3, as this is a typical advantage that can be obtained using covalent inhibitors [91]. A mechanism involving the covalent binding of electrophilic TR with a nucleophile exposed on the surface of the NLRP3 protein (Figure 14b), can therefore be hypothesised rather than a mechanism involving highly specific binding into a deep pocket of the target (typical of non-covalent small molecule inhibitors). This kind of binding could be the basis of the observed block of protein-protein interactions.

*Ex vivo* experiments that worked with patient-derived SFCs also demonstrated that TR (50 and 100  $\mu$ M) is effective in reducing NLRP3-mediated IL-1 $\beta$  release with no detectable effect on TNF secretion. Overall, TR exerts a protective effect in different models of NLRP3-driven acute inflammation.

TR (200 mg/kg; i.p.) efficiently suppressed IL-1 $\beta$  release and neutrophil recruitment when tested in a mouse model of peritonitis that had been evoked by MSU. In this model, its effect was 5 fold lower than the effect obtained after the administration of MCC950 (40 mg/kg; i.p.). After oral administration (200 mg/kg in CMC), TR was effective in a model of MSU-induced gouty arthritis and significantly reduced MSU-induced joint swelling and IL-1 $\beta$  levels in the joint tissue supernatant. At 100 mg/kg (oral administration), TR was also able to prevent body weight loss and prolong survival time in mice bearing the MWS-associated mutation *Nlrp3* (A350V*neoR*). TR was tested in mice that were fed with a HFD that simulates the metainflammatory condition typical of T2D in order to ascertain its effectiveness in chronic NLRP3-driven inflammation. TR was administered orally at 25 and 50 mg/kg to HFD-fed mice. TR decreased weight gain, in a dose-dependent manner, had no effect on food intake, prevented the elevation of fasting glucose concentration and ameliorated insulin sensitivity. Moreover, histological analyses revealed a marked decrease in liver steatosis in HFD mice treated with TR. It is worth noting that TR had no effect in control mice that were fed with a standard diet. An examination of inflammatory parameters associated with the NLRP3-induced metabolic disorders revealed reduced caspase-1

activation, and reduced production of IL-1 $\beta$  and TNF in the adipose tissue and liver of TR-treated mice. However, these *in vivo* effects were not significantly different in the two TR doses used in this study. Interestingly, the same results were obtained when the treatment with TR was initiated 14 weeks after the start of the HFD regimen. These experiments indicate that TR can be effective in reducing T2D-associated metabolic disorders via the inhibition of NLRP3 inflammasome signalling.

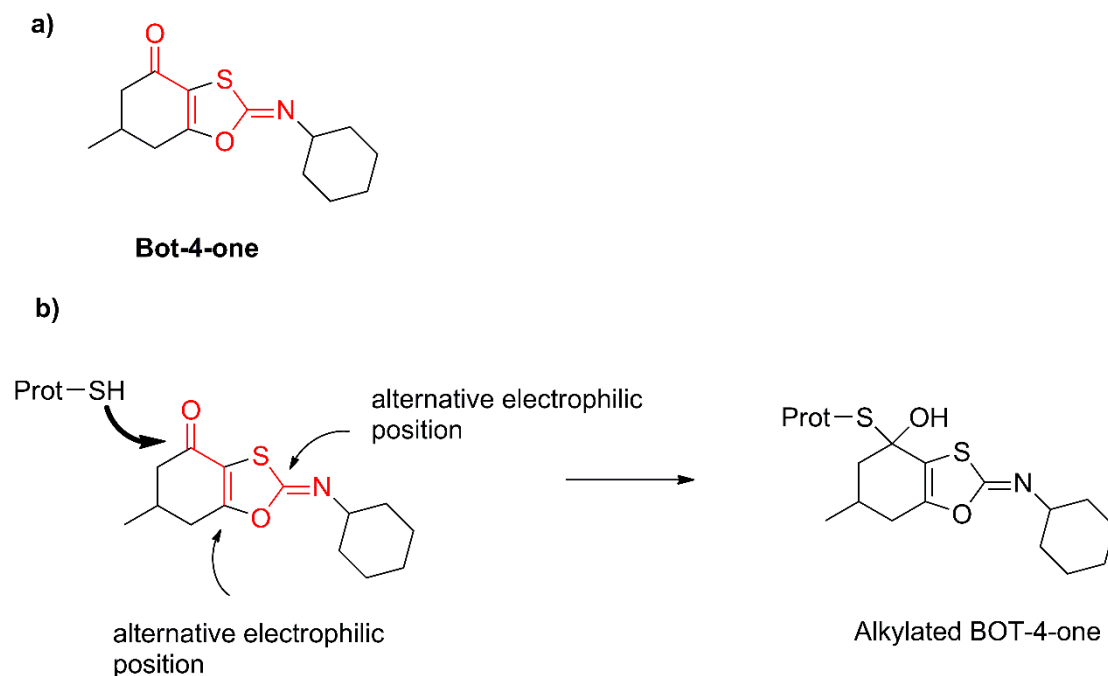
An analysis of results obtained while working with TR indicate that this drug can also act upstream of inflammasome signalling, as proven by its ability to prevent IL-6 release and pro-IL-1 $\beta$  expression, when administered to macrophages before LPS challenge. The *in vivo* effects of TR may be the result of multiple site targeting, rather than simple NLRP3 inflammasome inhibition.

## **12. (E)-(2-cyclohexylimino)-6-methyl-6,7-dihydro-5H-benzo[1,3]oxathiol-4-one (BOT-4-one)**

Early work on benzoxathiol-one derivatives identified this class of compounds as potential anti-psoriatic agents in the 1970s [132]. BOT-4-one (Figure 15a) is a benzoxathiole derivative developed from a series of anti-proliferative compounds that were originally reported on by Venkateswararao *et al.* in 2012 [133]. This compound was later investigated for its immunomodulatory activity in a dermatitis model [134]. BOT-4-one is an alkylating agent that provides the complete inhibition of IKK $\beta$  activity at a 30  $\mu$ M concentration. This action is reflected in the downmodulation of the NF- $\kappa$ B signalling pathway. *In silico* studies have shown that it is predicted to alkylate Cys179 in the activation loop of IKK $\beta$  kinase [134].

BOT-4-one has recently been reassessed as a potential NLRP3 inhibitor thanks to the discovery of NLRP3 inflammasome's sensitivity to electrophiles [135]. The authors found that BOT-4-one is able to dampen both canonical and non-canonical NLRP3 inflammasome activation. BOT-4-one dose-dependently (0.75 – 3  $\mu$ M) inhibited LPS/ATP-, LPS/nigericin-, and LPS/silica-induced caspase-1 activation, IL-1 $\beta$  release and pyroptosis (inhibition was less efficient when pyroptosis

was triggered by silica) in both BMDMs and THP-1 cells. The almost complete inhibition of these effects was reached at a 3  $\mu$ M concentration.



**Figure 16.** a) Molecular structure of BOT-4-one; b) predicted reactivity with Cys179 in IKK $\beta$  kinase (bold arrow) and alternative positions available for nucleophilic attack (plain arrows)

In BMDMs, BOT-4-one was observed to be somewhat more efficient than MNS and BAY 11-7082. BOT-4-one acts specifically against NLRP3, does not inhibit AIM2 and only partially inhibits NLRC4. The authors hypothesized that the effect on NLRC4 could be mediated by NLRP3-inhibition, as NLRP3 might be recruited as a coactivator to potentiate NLRC4 activation [136]. However, direct action on NLRC4, by BOT-4-one, cannot be ruled out and the possibility necessitates further study. In order to define the site of action of BOT-4-one, the authors verified its possible effects on pro-IL-1 $\beta$ , NLRP3 and ASC expression together with its effects on  $\alpha$ -tubulin acetylation and mitochondrial destabilization. None of these parameters were affected by BOT-4-

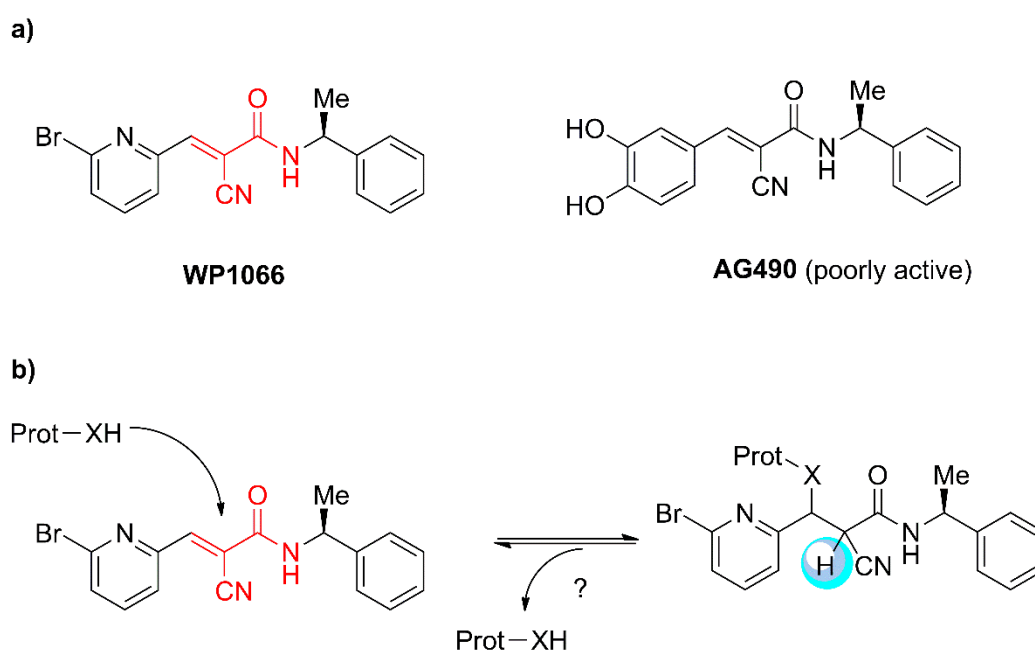
one treatment in macrophages. This compound was, however, able to attenuate the ATPase activity of recombinant NLRP3 (approx. 80 % reduction at 3  $\mu$ M) and suppress ASC-speck formation in BMDMs, both in a dose-dependent manner. These effects were attributed to BOT-4-one's ability to alkylate relevant nucleophilic residues in NLRP3, as demonstrated by its lack of action in the presence of an excess of L-cysteine. In an interesting finding, the authors highlighted the capacity of alkylating agent, such as BOT-4-one, MNS and BAY 11-7082, to up-regulate the ubiquitination of NLRP3. This effect and ATPase inhibition are independent of PKA activity, which is not affected by BOT-4-one or other known NLRP3 alkylating agents (MNS and BAY 11-7082). It is not clear whether this action is mediated through the inhibition of BRCC3 deubiquitinase (as found with compound G5 [53]), or through the promotion of NLRP3 ubiquitination. It is important to note that the alkylation of IKK $\beta$  was predicted to happen via the nucleophilic addition of Cys179 to the carbonyl group of BOT-4-one. However, in the case of NLRP3, a Michael addition to either positions 2 or 7a of BOT-4-one cannot be ruled out (Figure 15b). The anti-inflammatory action of BOT-4-one was validated *in vivo* in the described model of MSU-induced acute peritonitis in mice [14]. BOT-4-one (10 mg/kg, i.p.) significantly decreased IL-1 $\beta$  in the lavage fluid and reduced neutrophil infiltration in the inflammation site. Overall, BOT-4-one is another interesting covalent inhibitor in the arsenal of NLRP3-blocking molecules.

### 13. (E)-3-(6-bromopyridin-2-yl)-2-cyano-N-[(1S)-1-phenylethyl]prop-2-enamide (WP1066)

WP1066 (Figure 16a) is an acrylamide derivative that was originally synthesised and patented as an antiproliferative agent at the University of Texas [137].

WP1066 is an inhibitor of activated Janus kinases (JAKs) and acts by blocking the phosphorylation of the signal transducer and activator of transcription 3 (STAT3). This mechanism of action leads to growth inhibition and apoptosis induction in a variety of cancer cell lines, including those of acute myelogenous leukemia, malignant glioma and melanoma [138-140]. Honda *et al.* screened a library of 365 compounds with differentiated THP-1 cells that were treated with LPS/imiquimod, to induce

pro-IL-1 $\beta$  expression and NLRP3 priming and activation, with the aim of discovering small molecules that can regulate the release of mature IL-1 $\beta$  from cells. In this experiment, WP1066 was found to decrease IL-1 $\beta$  secretion (1-10  $\mu$ M) and provided almost complete suppression in the 2 – 10  $\mu$ M range. This compound was also validated using classical NLRP3 activators, such as nigericin, MSU and hypotonic medium. The same results were obtained working with peritoneal exudate cells (PECs). In this model, the activation of caspase-1 and the release of IL-1 $\beta$  were inhibited as well. Interestingly, authors reported that imiquimod, similarly to nigericin, was able not only to activate NLRP3, but also to induce differentiated THP-1 cell death, which was monitored using the LDH assay and propidium iodide (PI) staining.



**Figure 16.** a) Molecular structure of WP1066 and its analogue AG490; b) predicted reactivity with nucleophilic residues: the reversibility of the reaction depends on the acidity of the  $\alpha$ -hydrogen (highlighted in cyan).

In this system, WP1066 completely abolished cell death at 10  $\mu$ M, while only a weak effect was obtained when working with the caspase-1 inhibitor Ac-YVAD-CMK. In PECs, WP1066 exerted the same anti-pyrototic action as seen in THP-1. Notably, the caspase-1 inhibitor was inefficient in preventing imiquimod-induced cell death in this case. These results suggest that either imiquimod triggers a type of cell death that is different to pyroptosis, or that WP1066 exerts its action by targeting multiple signalling pathways rather than the NLRP3/caspase-1 pathway alone. The authors decided to study the influence of STAT3 signalling on inflammasome activation by monitoring the phosphorylation of STAT3 at Tyr705 (P-Y705) and Ser727 (P-S727) in order to identify possible alternative target(s) for WP1066. Interestingly, the authors noted that neither imiquimod nor the other NLRP3 activators used were able to promote (or further activate) the phosphorylation of Y705. WP1066 slightly attenuated the formation of STAT3 P-Y705 in PMA-differentiated and LPS-stimulated THP-1 cells. The known JAK/STAT inhibitors, ruxolitinib and AG490 (a close analogue of WP1066, figure 16a), failed to suppress, or only slightly affected IL-1 $\beta$  release or the pyrototic process. Collectively, these results indicate that STAT3 signalling is not involved in the release of IL-1 $\beta$ , and that the NLRP3 and STAT3 signalling pathways are not likely to interfere with each other in THP-1 cells. Rather intriguingly, WP1066 (10  $\mu$ M) was cytotoxic in undifferentiated THP-1 cells and in A172 and T98G, two glioma cell lines; this cytotoxicity was not dependent on STAT3 inhibition.

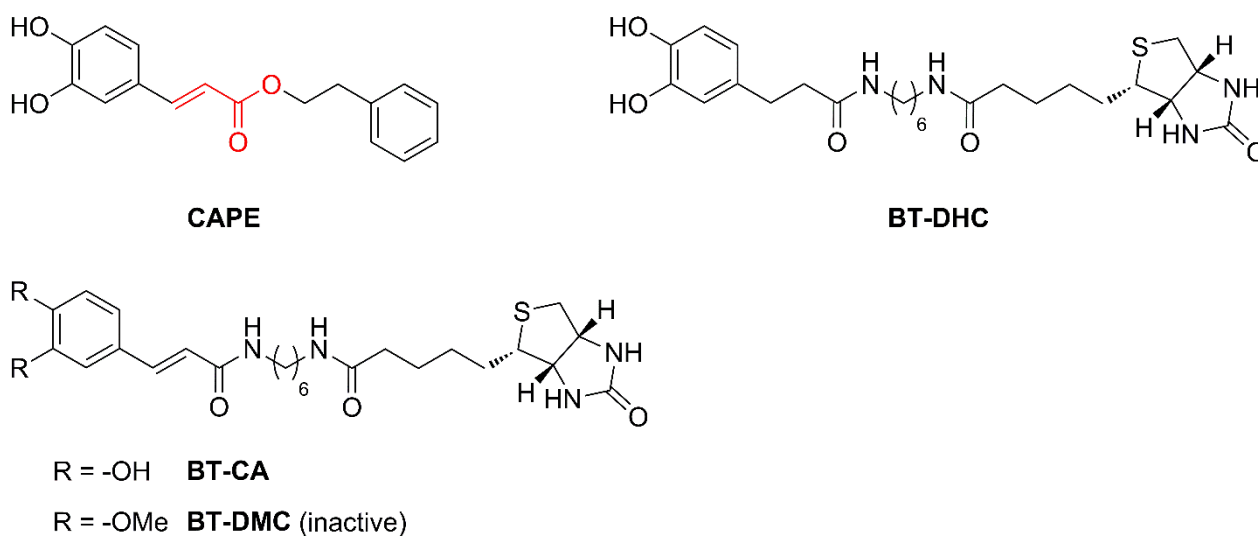
Mechanistically speaking, WP1066 is an alkylating agent, which can react with biological nucleophiles. Its structure contains a double electrophilic moiety, namely an acrylamide, and an acrylonitrile. The adduct obtained following Michael reaction with nucleophiles may be stable, leading to covalent irreversible inhibition, or can revert to the starting compounds, leading to covalent reversible inhibition [141]. The reversibility of the reaction largely depends on the acidity of the hydrogen  $\alpha$  to the nitrile and the amide functionality (Figure 16b), and on the cellular microenvironment. Since WP1066 was able to induce JAK2 protein degradation [138], it is likely that WP1066 acts as an irreversible alkylating agent on JAK2 and this activity may be translated to



NLRP3 when it is the target. Some aspects of the mechanism of NLRP3 inhibition by WP1066, as well as its *in vivo* efficacy, still require further investigation.

#### 14. Caffeic acid phenylethyl ester (CAPE)

Lee *et al.* have selected caffeic acid phenylethyl ester (CAPE; Figure 17) from a library of compounds of natural origin as part of their search for new molecules for the treatment of gouty arthritis. CAPE was tested in BMDMs and THP-1 cells that had been primed with LPS and stimulated with MSU crystals [142]. In this model, CAPE (0.5 – 10  $\mu$ M) was able to inhibit ASC speck formation, caspase-1 activation and IL-1 $\beta$  secretion in a dose-dependent manner, albeit with slightly lower efficacy in human macrophages than in BMDMs. CAPE also inhibited the release of IL-1 $\beta$  in BMDMs after a challenge with classical stimuli, such as ATP and nigericin.



**Figure 17.** Molecular structures of caffeic acid phenylethyl ester (CAPE) and its biotinylated analogues that were used for mechanism of action studies.

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These results were further validated in an *in vivo* mouse model of acute inflammation. CAPE (30 mg/kg) was administered orally after air pouches were generated on the backs of the mice. One hour later, the NLRP3 inflammasome was activated by MSU injection (3 mg/mL/mouse) into the air pouch. In this experimental model, CAPE decreased caspase-1 activity, IL-1 $\beta$ , and IL-18 secretion in the air pouch exudates, inhibiting myeloperoxidase (MPO) activity and neutrophil infiltration into the site of inflammation. Similar results were observed in a gouty arthritis model obtained by MSU injection into the hind foot of mice. In this model, CAPE (30 mg/kg per os) was able to decrease caspase-1 activation, IL-1 $\beta$ , IL-18, MPO activity, neutrophil infiltration and foot pad thickness, while no TNF inhibition was detected. The authors conducted mechanism of action studies in 293T cells that overexpressed the inflammasome components NLRP3, ASC and caspase-1, both alone and in combination, by measuring the ability of assembled inflammasome to activate the response of luciferase reporter gene (iGLuc). With this assay, the authors excluded the possibility of direct action on caspase-1, and identified ASC as a preferential target for CAPE. The production of three biotinylated analogues of CAPE, namely BT-CA (active), BT-DHC (active), and BT-DMC (inactive) (Figure 17) allowed the BT-CA-ASC and BT-DHC-ASC complexes to be isolated. Surface Plasmon Resonance (SPR) studies further confirmed CAPE's direct binding to ASC. Its ability to bind ASC means that CAPE was also able to inhibit the activation of AIM2 inflammasome, which also requires ASC for its assembly.

This study shows that ASC is a possible target for CAPE. SPR experiments with different concentrations of CAPE, followed by washout, also demonstrated that *in vitro* binding between the isolated ASC protein and CAPE appears to be reversible. This was further supported by pull down experiments in cell lysates conducted with BT-DHC, which is the biotinylated analogue of CAPE that is deprived of the Michael acceptor moiety. BT-DHC was still able to interact with ASC under these conditions. From these results, we might conclude that the free catechol ring is fundamental for the interaction between CAPE and the ASC component of the inflammasome. However, while this study demonstrates the reversible binding of CAPE to ASC, we feel that CAPE may still

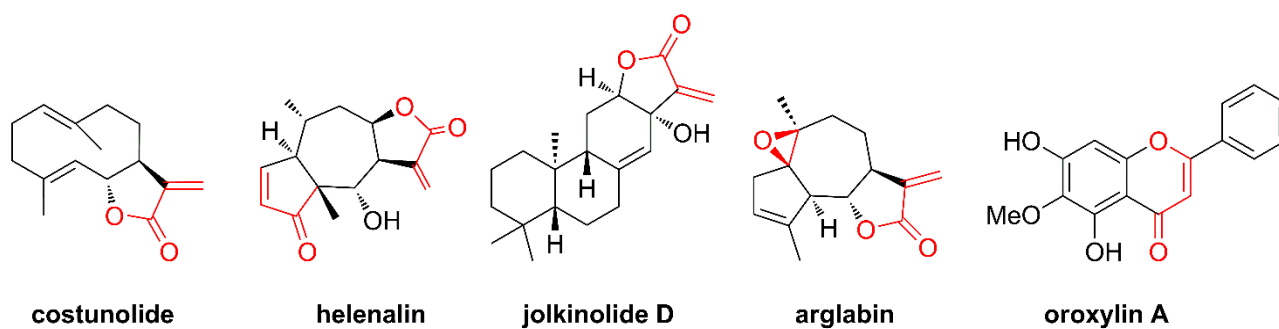
irreversibly interact with either NLRP3 or other proteins that are involved in the NF- $\kappa$ B signalling pathway [143].

## **15. Other electrophilic NLRP3 inhibitors**

We have described, in the previous sections, the mode of action of the most frequently studied compounds and classes of compounds that act (or are likely to act) as irreversible NLRP3 inhibitors. While other compounds that can inhibit NLRP3 signalling have been described, they suffer from a lack of deep investigation into their modes of action, or they act by irreversibly inhibiting multiple targets beyond the NLRP3 inflammasome. In the following pages, we will briefly discuss some compounds that fall into the scope of this review and that are cited as NLRP3 inhibitors in recent literature.

Some natural derivatives have been found to irreversibly inhibit NLRP3 signalling, and include parthenolide (Figure 4) [88], costunolide, helenalin [144], jolkinolide [145] and arglabin [146] (Figure 18). These natural derivatives all possess a  $\gamma$ -methylene lactone moiety that is known to react with a variety of nucleophiles, such as the nitrogen atom in alanine, the ring nitrogen in histidine, and the sulphur atom in cysteine and GSH [145]. Their high reactivity means that  $\gamma$ -methylene lactone derivatives can trap cellular nucleophiles that are involved in a number of different signalling pathways. Consequently, it is not surprising that they exert anti-inflammatory effects through the inhibition of multiple targets along the NF- $\kappa$ B/NLRP3/caspase-1 axis [147-149]. None of these can be regarded as selective NLRP3 inhibitor. However, the anti-inflammatory and antiatherogenic activity of arglabin in APOE.Ki mice, susceptible to develop atherosclerosis under HFD regimen, looks particularly promising [146].

The flavonoid derivative Oroxylin A (Figure 18) has recently proven that it can inhibit dextran sulfate sodium (DSS)-induced colitis in mice via both the inhibition of the NF- $\kappa$ B-mediated expression of NLRP3 and via direct action exerted on the NLRP3 assembly [150].



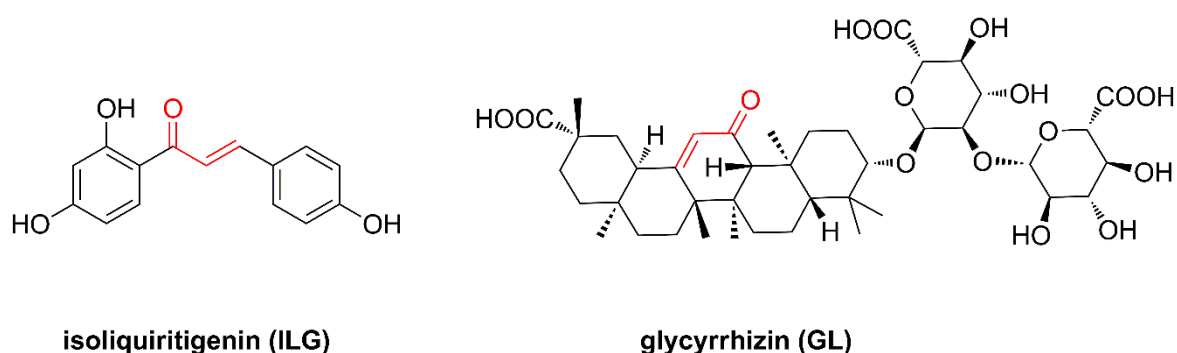

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**Figure 18.** Molecular structures of representative natural derivatives that act as multi-target anti-inflammatory agents and NLRP3 inhibitors.

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Chalcones have been widely studied because of their ability to react, in a Michael fashion, with cysteines in proteins and form covalent protein-chalcone adducts, thereby irreversibly blocking protein function [151,152]. Isoliquiritigenin (ILG), a flavonoid with a chalcone substructure that can be isolated from *Glycyrrhiza uralensis* (Figure 19), has shown interesting NLRP3 signalling inhibition. ILG was initially characterized as an inhibitor of TLR4 activation/NF- $\kappa$ B activity, and of mitogen-activated protein kinase (MAPK) activity. However, its ability to inhibit NLRP3 activation, independently from TLR4 antagonism, has recently been demonstrated [153]. In BMDMs, ILG inhibited NLRP3 inflammasome-induced IL-1 $\beta$  production and caspase-1 activation to a higher extent than parthenolide and glyburide. *Ex vivo* and *in vivo* analyses revealed that ILG reduced HFD-induced IL-1 $\beta$  and caspase-1 production from white adipose tissue, and lowered weight gain, hypercholesterolemia, hepatic steatosis, adipose tissue inflammation and insulin resistance. Some of these effects are shared by glycyrrhizin (GL), a saponine derivative that contains an electrophilic function that is isolated from plants of the *Glycyrrhiza* genus (Figure 19). However, GL action is not specific against NLRP3, as GL can also inhibit AIM2 inflammasome, albeit at higher doses.

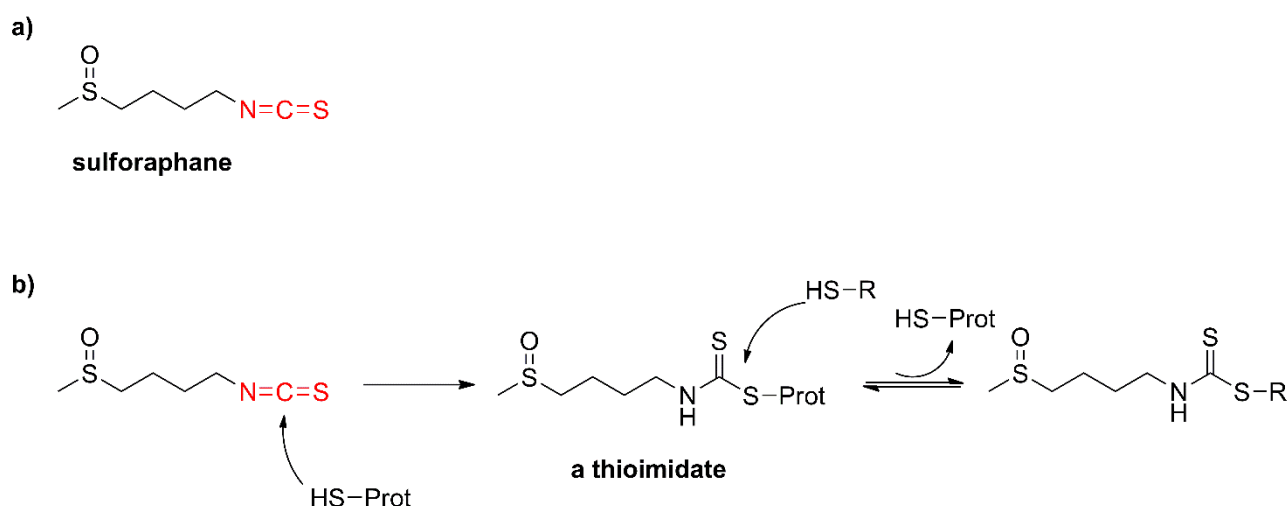
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**Figure 19.** Molecular structures of isoliquiritigenin (ILG) and glycyrrhizin (GL).

Another natural compound that can inhibit the NLRP3 inflammasome is sulforaphane (*R*-1-isothiocyanato-4-methylsulfinylbutane) [154,155], an isothiocyanate derivative that is isolated from cruciferous vegetables (Figure 20a). Sulforaphane has long been known for its anti-inflammatory and cytoprotective effects [156]. Its anti-inflammatory effects have been ascribed to its ability to activate transcription factor Nrf2. Keap1 has recently been isolated and identified to be an inhibitory regulator of Nrf2 that binds to the N-terminal Neh2 domain of Nrf2. The cysteine-rich Keap1 protein (27 Cys residues) activates Nrf2 following its reaction with electrophiles to form covalent adducts, which stabilizes Nrf2 and results in enhanced nuclear Nrf2 levels. As a matter of fact, sulforaphane stabilizes and activates the Nrf2 transcription factor by reacting with the cysteine residues of its repressor, Keap1 [157]. Thiols react with sulforaphane via addition to the isothiocyanate carbon to yield S-thionoacyl adducts (Figure 20b) as demonstrated for the Keap1 protein. The formed adduct is stable in physiological conditions and can only be cleaved in the presence of other nucleophiles, via a transacylation reaction or by protease-mediated hydrolysis [158]. Additional evidence suggests that sulforaphane may react directly with other cellular targets, such as TLR4 and tubulin, in a thiol-dependent manner, via the formation of covalent bonds [159-161].

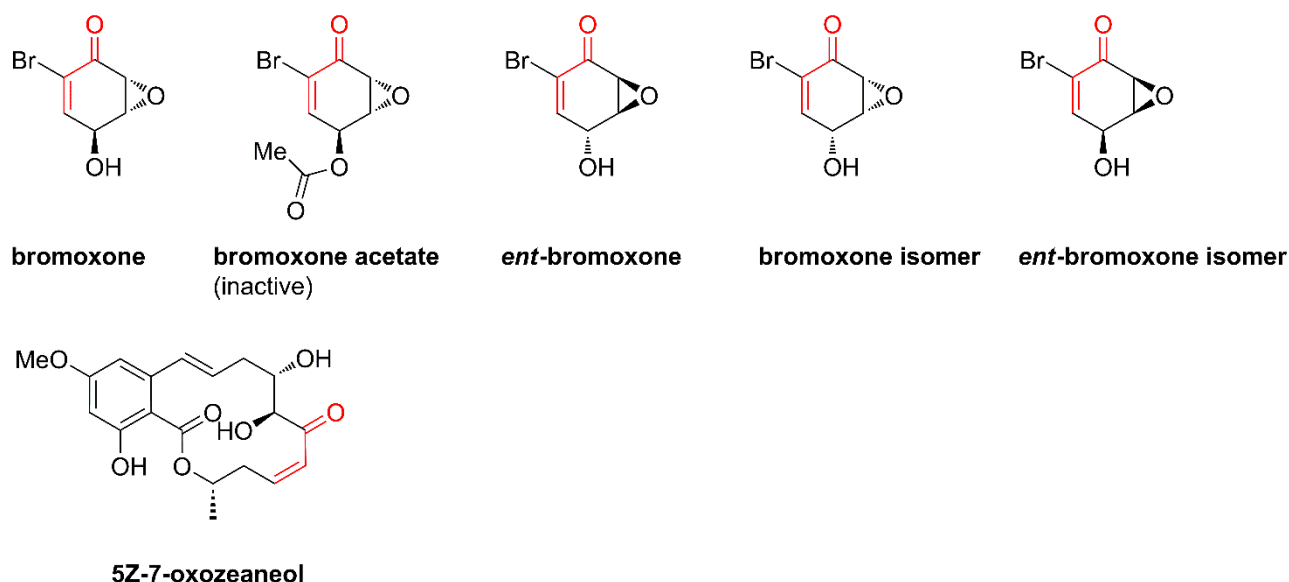
Recent studies have demonstrated that sulforaphane is able to prevent the activation of multiple inflammasomes, namely NLRP3, NLRP1, NLRC4 [154,155]. The ability of sulforaphane to attenuate the activation of AIM2 inflammasome is still controversial. Its action is not dependent on either Nrf2 activation or ROS scavenging, and may occur through a direct covalent interaction with the inflammasome proteins, thus preventing conformational changes that are essential for inflammasome activation and assembly. Interestingly, sulforaphane proved itself to be active in an *in vivo* model of MSU-induced peritonitis as it was able to reduce IL-1 $\beta$  maturation following the i.p administration of 25 mg/kg twice in four hours [154,155].



**Figure 20.** a) Molecular structure of sulforaphane. b) Reaction of sulforaphane with thiol containing proteins and trans-thioacylation.

Bromoxone, together with some of its synthetic analogues (Figure 21a) [89], are other non-specific inflammasome inhibitors. They are able to decrease the activation of NLRP3, NLRP1, and AIM2 inflammasomes in a NF- $\kappa$ B-independent manner. However, bromoxone also decreases pro-IL-1 $\beta$

expression by interfering with the NF- $\kappa$ B pathway upstream of IKK $\beta$ -kinase. In the same work, Gong *et al.* identified 5Z-7-oxozeaneol (Figure 21b) as an NLRP3 inhibitor and found that it shares similar properties to bromoxone.



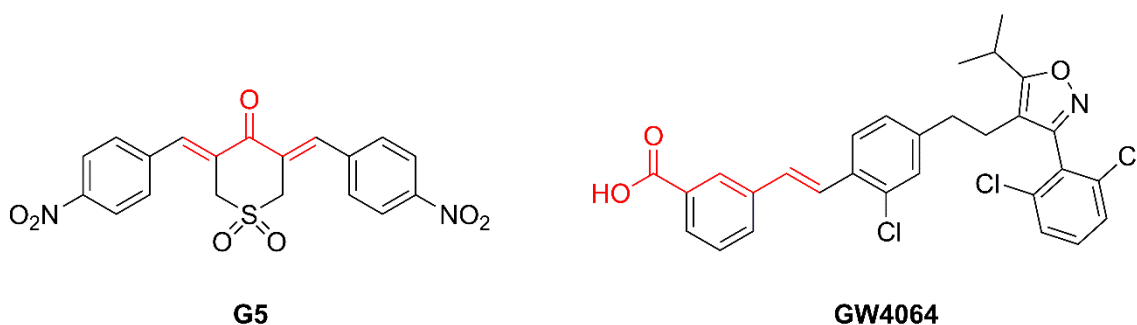
**Figure 21.** a) Molecular structure of bromoxone and its analogues that have been identified as NLRP3 inhibitors; b) Molecular structure of 5Z-7-oxozeaneol.

Several negative regulators, which either act directly or via the post-translational modification of the proteins that make up the NLRP3 inflammasomes, have been identified. As mentioned above, deubiquitinase BRCC3 plays an essential role as one of the negative regulators of the NLRP3 inflammasome.

In 2013, Py *et al.* discovered that G5 (4H-thiopyran-4-one, tetrahydro-3,5-bis[(4-nitrophenyl)methylene]-1,1-dioxide; Figure 22), which is a non-selective isopeptidase inhibitor that was originally developed as an anticancer agent [162], was able to inhibit NLRP3 activation via the inhibition of BRCC3 activity [53]. G5 possess a cross-conjugated  $\alpha,\beta$ -unsaturated dienone structure

bearing two sterically accessible electrophilic  $\beta$ -carbons, meaning that it can therefore act as a Michael acceptor for the targeting of nucleophiles, such as the catalytic cysteine of several isopeptidases. Its electrophilic properties mean that G5 is not selective for BCCR3 as it is also able to inhibit ubiquitin carboxy-terminal hydrolases UCHL1, UCHL5 and the ubiquitin-specific protease USP2 and USP18 as well [163].

GW 4064 (Figure 22) is a small molecule that is known as an agonist of the nuclear farnesoid X receptor (FXR), through which bile acids modulate glucose metabolism. Activation of the FXR receptor has recently been demonstrated to negatively modulate NLRP3 inflammasome activation, and TGR5 (a G-protein-coupled bile acid receptor). The connection between bile acids and inflammasome regulation is yet not completely understood [164,165] and the mechanism of NLRP3 inhibition by GW4064 has not been well defined either. Liu *et al.* have reported on the inhibitory effect of GW4064 on LPS-induced ileum injury in mice, and suggested that a mechanism involving TLR4/MyD88 downregulation may be involved [166]. However, Xie *et al.* have demonstrated that GW 4064 can inhibit NLRP3 inflammasome activation in a FXR-independent manner, by decreasing the ubiquitination and oligomerisation of ASC without altering NLRP3 ubiquitination or preventing NLRP3-ASC interactions [164]. The administration of GW 4064 inhibited IL-1 $\beta$  and IL-18 release in BMDMs, in a dose-dependent manner, without interacting with the priming step of NLRP3. Nevertheless, when GW 4064 was administrated before LPS, it also inhibited NF- $\kappa$ B activation.





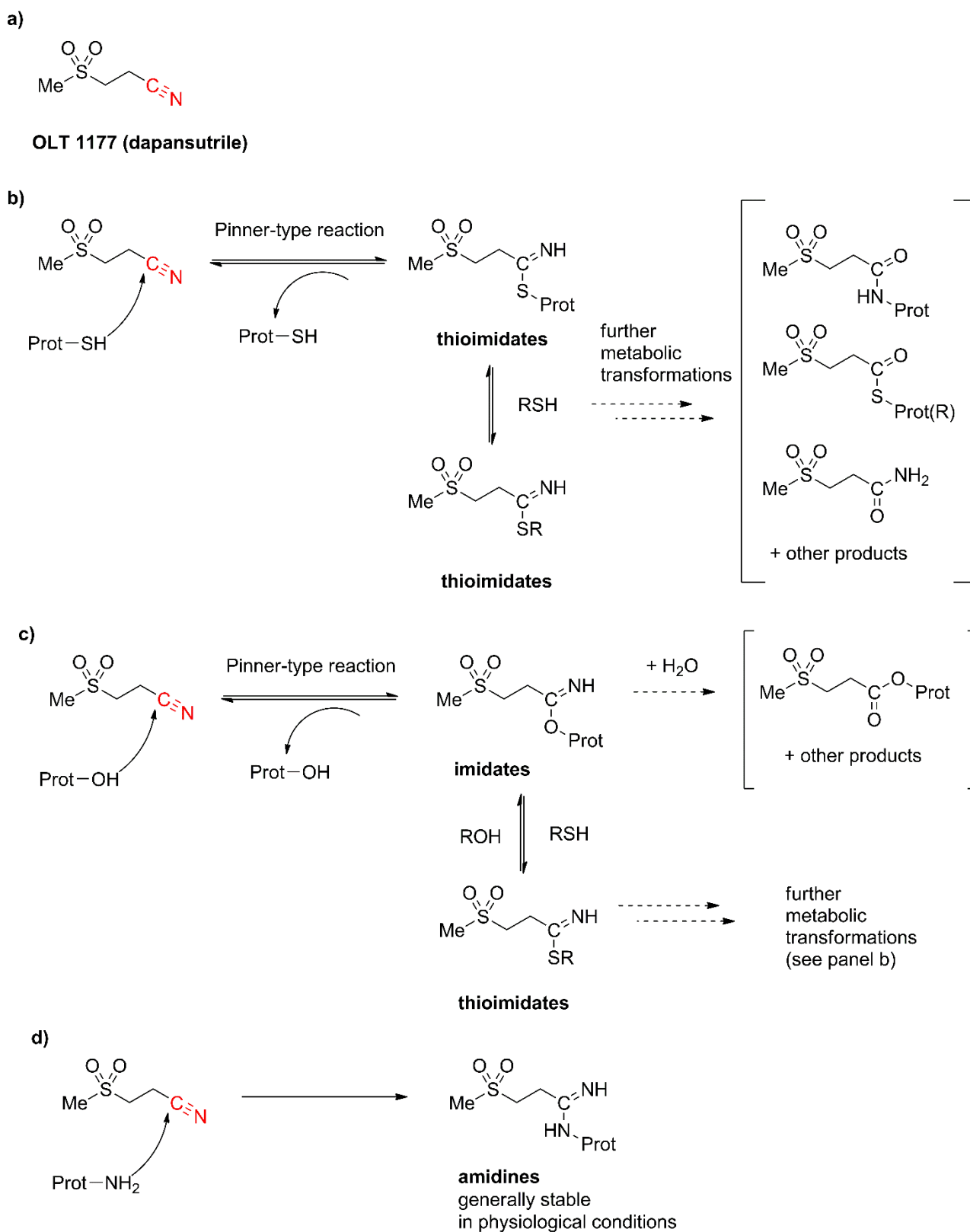
**Figure 22.** Molecular structures of G5 and GW4064.

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Another interesting NLRP3 inhibitor is OLT1177 (3-methylsulfonylpropionitrile), which is also known as dapansutride (Figure 23a). Marchetti *et al.* have recently demonstrated that this small molecule potently inhibits NLRP3 activation, as well as IL-1 $\beta$  and IL-18 release from human macrophages and neutrophils. OLT1177 directly inhibits NLRP3 ATPase activity and prevents NLRP3-ASC and NLRP3-caspase-1 interactions from occurring [167]. OLT1177 is currently under development as a drug by Olatec Therapeutics [168]. OLT1177 was proven to be safe in healthy volunteers and is now undergoing phase 2b clinical trials for possible development into a new drug for the treatment of osteoarthritis of the knee [169].

Nitrile derivatives have already been successfully exploited as covalent drugs. An example can be found in saxagliptin, a marketed DPP-IV inhibitor that acts through the covalent binding of catalytic Ser630 in the protease binding pocket [170]. As OLT1177 is a nitrile derivative, it can react with biological nucleophiles, such as thiols, alcohols and amines in a Pinner-type reaction to form thioimidates, imidates and amidines, respectively [171,172]. Depending on the type of product generated, the covalent bond formed can be relatively unstable, leading to it either regenerating the parent compound, or being converted into other adducts via reaction with nearby nucleophiles (Figure 23b-d). The rate of hydrolysis/conversion depends on the biological microenvironment and the accessibility of the formed bond by water. Studies into the reactivity of the nitrile group in OLT1177 are currently not available and, consequently, a final classification of OLT1177 as a covalent/non-covalent or reversible/irreversible inhibitor is not possible. However, it is the opinion of this review's authors that its potent NLRP3 inhibition may arise from covalent inhibition at the protein level.

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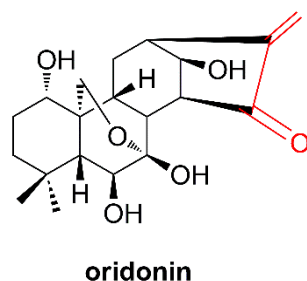


**Figure 23.** a) Molecular structure of OLT1177; b-d) putative reaction pathways of OLT1177 with (b) sulphur-, (c) oxygen-, (d) and nitrogen-based nucleophiles.

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An electrophilic natural ent-kaurane diterpenoid derivative, oridonin (Figure 24) which is isolated from *Rabdosia rubescens*, was demonstrated to inhibit NLRP3 signalling while this review was being prepared [173]. Oridonin was previously described as a 5'-adenosine monophosphate-activated protein kinase (AMPK) inhibitor that can interfere with glucose metabolism and induce autophagy in colorectal cancer cells [174]. The action of oridonin on NLRP3 is mediated by covalent binding to Cys279 in the NACHT domain of NLRP3. This binding prevents the NLRP3-NEK7 interaction from occurring and thereby inhibits NLRP3 activation and assembly. Oridonin is active at 20 mg/kg in mouse models of peritonitis and gouty arthritis, it also counteracts the inflammatory effect triggered with a HFD regimen when administered to mice at 3 mg/kg for six weeks restoring insulin sensitivity and decreasing intracellular lipid accumulation in liver [173].

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**Figure 24.** Molecular structure of oridonin.

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In table 1 we report a summary of *in vitro* data of selected molecules in order to help the reader having an overview about covalent NLRP3 inhibitors described in this review. Of note, in completing table 1 some data have been extrapolated from figures and graphs, therefore, little approximation might be present.

## 16. Concluding remarks

The discovery of new agents that can block NLRP3 inflammasome signalling holds great promise for the development of a new class of anti-inflammatory and immunomodulatory drugs. This will thus pave the way for new pharmacological options for the treatment of numerous pathological conditions with unmet needs. The research of effective and selective NLRP3 inflammasome inhibitors is still a highly challenging task for biologists, pharmacologists and medicinal chemists. A great deal of progress has recently been made in the elucidation of NLRP3 activation and signalling mechanisms. However, there is still a lack of detail as to its molecular 3D structure, which thus complicates the discovery of drugs that can act directly on the protein target.

Early observations suggested that the use of electrophilic compounds may be a good starting point from which to develop NLRP3 blocking agents [103]. Electrophilic compounds are able to covalently bind to cysteine-rich NLRP3, and thereby irreversibly or reversibly block target protein activity, depending on the electrophilic warhead employed. As described in this review, this strategy has proven to be generally successful, and numerous electrophilic NLRP3 inhibitors have been reported. However, the use of covalent inhibitors entails some precautions, because, even if their reactivity is appropriately tuned, they can give non-specific reactions with other nucleophiles in proteins. Most of the off-target interactions that occur with electrophilic NLRP3 inhibitors have been identified within the NF- $\kappa$ B and Keap1/Nrf2 pathways. From a therapeutic point of view this is not necessarily a disadvantage, as NF- $\kappa$ B inhibition can act synergically with NLRP3 inhibition and lead to enhanced anti-inflammatory activity *in vivo*.

The discovery of new small molecules that act as covalent NLRP3 inhibitors must therefore take into account the possibility that interactions may occur with other targets. A recently proposed tool kit, which is made up of well-characterized molecules endowed with nanomolar potency against different points of the inflammasome pathway, can be of help in the discovery and biological validation of new small molecule acting as specific NLRP3 inhibitors [175]. Moreover, ligands

blocking NLRP3 through different mechanisms could help in investigating new hypothesis and look for the ideal inhibitory strategy. Non-covalent inhibitors (e.g. MCC950), covalent/non covalent inhibitors able to block NLRP3 ATPase activity (e.g. INF39, CY-09, OLT1177), and covalent inhibitors devoid of this last property (e.g. oridonin) might represent useful tools toward the choice of the best NLRP3 inhibition strategy in different contexts.

There are no univocal winning strategies for the development of new covalent NLRP3 inhibitors. A possibility may be the development of targeted covalent inhibitors that are specific for NLRP3. However, this drug-design strategy is currently hampered by the limited structural knowledge that we have of the target.

Another intriguing approach could be the inhibition of NLRP3 ATPase activity, as all the small molecules that inhibit it have shown an in-cell ability to prevent IL-1 $\beta$  release. When developing such a class of agents, allosteric inhibitors, rather than agents that compete with ATP for its binding pocket, would appear to be preferable if selective NLRP3 inhibitors are to be obtained.

**Table 1.** Summary of biological effects relevant to NLRP3 inflammasome inhibition for selected NLRP3 inhibitors described in this article.

Compound (rev/irrev)	Main mechanism of action	Selectivity vs other inflammasomes	IL-1 $\beta$ inhibition % ( $\mu$ M)	NLRP3 ATPase inhibition % ( $\mu$ M)	Pyroptosis Inhibition % ( $\mu$ M)	Other target(s) <sup>a</sup>	Cytotoxicity TC <sub>50</sub> $\pm$ SE ( $\mu$ M)	Ref
<b>BAY</b> (irrev)	Inhibition of NLRP3 ATPase	Partial inhibition of NLRC4	>80 % (10-100 $\mu$ M) <sup>b</sup>	60 % (10 $\mu$ M)	60 % (10 $\mu$ M) <sup>b</sup>	PTPs; IKK $\beta$ ;	1.5 $\pm$ 0.3 <sup>c</sup>	[88] [97-100] [102] [167]
<b>MNS</b> (irrev)	Inhibition of NLRP3 ATPase	selective	2 –100 % (0.5–10 $\mu$ M) <sup>b</sup>	35–75 % (1–10 $\mu$ M)	85 % (5 $\mu$ M) <sup>b</sup>	Syk and Src kinases	3.2 $\pm$ 0.8 <sup>c</sup>	[90] [102]
<b>INF39</b> (irrev)	Inhibition of NLRP3 ATPase	NT	60-80 % (10-100 $\mu$ M) <sup>b</sup>	29-52 % (50-100 $\mu$ M)	34 % <sup>d</sup> (10 $\mu$ M)	<i>NF-kB</i>	Non toxic up to 100 $\mu$ M <sup>c</sup>	[102]
<b>INF58</b> (irrev)	Inhibition of NLRP3 ATPase	NT	65 % (20 $\mu$ M) <sup>b</sup>	50 % (74 $\mu$ M)	10-64 % <sup>d</sup> (1-100 $\mu$ M)	-	Non toxic up to 100 $\mu$ M <sup>c</sup>	[95]
<b>NBC6</b> (irrev)	Inhibition of NLRP3 assembly	Selective up to 10 $\mu$ M. AIM2 also targeted at higher conc.	99 % (10 $\mu$ M) <sup>e</sup>	NT	NT	-	Non toxic up to 100 $\mu$ M <sup>f</sup>	[110] [111]
<b>CY-09</b> (rev ?)	Inhibition of NLRP3 ATPase	selective	40–85 % (1–10 $\mu$ M) <sup>b</sup>	40–85 % (0.1–1 $\mu$ M)	80 % (10 $\mu$ M) <sup>g</sup>	-	Not reported	[113] [173]
<b>DiMF</b> (irrev)	Unknown; multiple targets ?	NT	20–65 % (0.1–10 $\mu$ M) <sup>d</sup>	inactive	65 % (10 $\mu$ M) <sup>d</sup>	Keap-1/NRF2; HCA2 ?	95.5 $\pm$ 1.2 <sup>c</sup>	[102] [125] [126]
<b>Tranilast</b> (irrev ?)	Inhibition of NLRP3- NLRP3 and NLRP3- ASC interactions	selective	75 % (100 $\mu$ M) <sup>d</sup>	inactive	inactive	NF- $\kappa$ B pathway	Not reported	[130] [131]
<b>BOT-4-one</b> (irrev)	Inhibition of NLRP3 ATPase	Partial inhibition of NLRC4	35-99 % (0.75–3 $\mu$ M) <sup>b</sup>	50–80 % (1.5–3 $\mu$ M)	35-99 % (0.75–3 $\mu$ M) <sup>b</sup>	IKK $\beta$ ; NF- $\kappa$ B Akt signaling; NLRP3 ubiquitinylation	Non toxic up to 3 $\mu$ M <sup>c</sup>	[134] [135]
<b>WP1066</b> (irrev ?)	Unknown; Alkylation of NLRP3 ?	NT	80–100 % (1–10 $\mu$ M) <sup>h</sup>	NT	99 % (10 $\mu$ M) <sup>e</sup>	JAK-STAT3	<10 $\mu$ M <sup>c</sup>	[140]
<b>CAPE</b> (irrev ?)	Block of NLRP3- ASC interaction	Partial inhibition of AIM2	30-100 % (0.5–10 $\mu$ M) <sup>b</sup>	NT	NT	<i>NF-kB</i>	Non toxic up to 10 $\mu$ M <sup>i</sup>	[142]
<b>ILG</b> (irrev ?)	Unknown	selective	70–95 % (1-10 $\mu$ M) <sup>b</sup>	NT	inactive	TLR4/MD-2; NF- $\kappa$ B; MAPK; JNK1/2	Non toxic up to 10 $\mu$ M <sup>i</sup>	[153]
<b>SFN</b> (irrev ?)	unknown	NLRP1, NLRC4 AIM2 ?	100 % (5–50 $\mu$ M) <sup>b</sup> by Western blot	NT	100 % NLRP1- mediated pyroptosis (100 $\mu$ M)	Keap1/NRF2; TLR4	Non toxic up to 100 $\mu$ M <sup>i</sup>	[154] [155]

<b>Br</b> (irrev)	Multiple targets	AIM2, NLRP1	50 % (0.17 $\mu$ M) <sup>d</sup>	NT	85 % (2.5 $\mu$ M) <sup>d</sup>	<i>NF-<math>\kappa</math>B</i>	Non toxic at 2.5 $\mu$ M <sup>c</sup>	[89]
<b>Parth</b> (irrev)	NF- $\kappa$ B / NLRP3 / caspase-1 axis	Non selective NLRP1, NLRC4, AIM2	20-100 % (0.01-10 $\mu$ M) <sup>d</sup>	23 % (10 $\mu$ M)	80 % (1–20 $\mu$ M) <sup>b</sup>	NF- $\kappa$ B (IKK $\beta$ , p65); Caspase-1	20.1 $\pm$ 5.5 <sup>l</sup>	[88] [103] [125]
<b>OLT1177</b> (rev ?)	Inhibition of NLRP3 ATPase; block of NLRP3-ASC, NLRP3-caspase 1 interactions, block of NLRP3 assembly	selective	60 % (0.0001-10 $\mu$ M) <sup>m</sup>	60 % (1 $\mu$ M)	25–40 % (0.001–10 $\mu$ M) <sup>m</sup>	<i>Src; Fyn; HcK; STAT3; NF-<math>\kappa</math>B (in vivo)</i>	Non toxic in healthy volunteers up to 1 g/kg	[167]
<b>Oridonin</b> (irrev)	Block of NLRP3- NEK7, NLRP3-ASC interactions, NLRP3 assembly	selective	100 % (2 $\mu$ M) <sup>b</sup>	inactive	70-95 % (0.5–2 $\mu$ M) <sup>g</sup>	NF- $\kappa$ B; <i>MAPK</i>	Not reported	[173]

<sup>a</sup> Direct effects reported in literature are written in plain text, when experimental data cannot discriminate between direct or downstream effects, italic is used; <sup>b</sup>in LPS/ATP-stimulated BMDMs; <sup>c</sup>in THP-1 cells; <sup>d</sup>in PMA-treated and LPS/ATP-stimulated THP-1; <sup>e</sup>in PMA-treated and LPS/nigericin-stimulated THP-1; <sup>f</sup>in HEK293 and HepG2 cells; <sup>g</sup>in LPS/nigericin-stimulated BMDMs; <sup>h</sup>in PMA-treated and LPS/imiquimod-stimulated THP-1; <sup>i</sup>in BMDMs; <sup>l</sup>in HK-2 cells; <sup>m</sup>in LPS/ATP-stimulated murine macrophages J774A.1

Abbreviations: irrev= irreversible; rev = reversible; NT = not tested; SFN = sulforaphane; ILG = isoliquiritigenin; parth = parthenolide; BAY = BAY 11-7082; Br = bromoxone; DiMF = dimethylfumarate; PTPs = protein tyrosine phosphatases; IKK = inhibitor of nuclear factor kappa-B kinase; HCA2 = hydroxyl carboxylic acid receptor 2; NF- $\kappa$ B = Nuclear factor kappaB; MAPK = Mitogen-activated protein kinase; TLR4 = Toll-like receptor4; Keap1/NRF2 = Kelch-like ECH-associated protein 1/ Nuclear factor (erythroid-derived 2)-like 2; STAT3 = signal transducer and activator of transcription protein3; Src = Proto-oncogene tyrosine-protein kinase Src; Fyn = Proto-oncogene tyrosine-protein kinase Fyn; HcK = Tyrosine-protein kinase HCK ; JNK1/2 = c-Jun N-terminal kinase 1/2; JAK = Janus kinase; IKK $\beta$  = inhibitor of nuclear factor kappa-B kinase subunit beta; Akt = AKT serine/threonine kinase.

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